Characterization of different types of radiation- and pressure-induced DNA damage in *Bacillus subtilis* spores and their global transcriptional response during spore germination

Von der Fakultät für Lebenswissenschaften

der Technischen Universität Carolo-Wilhelmina

zu Braunschweig

zur Erlangung des Grades eines

Doktors der Naturwissenschaften

(Dr. rer. nat.)

genehmigte

D i s s e r t a t i o n

von

Ralf Möller

aus Suhl / Deutschland

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aus Suhl

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2. Referent:	Prof. Dr. Erko Stackebrandt
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“All truths are easy to understand once they are discovered; the point is to discover them.”
(Galileo Galilei, 1564 - 1642)

This thesis is dedicate to my mother
(Marion Möller, 1949-2006).

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Abbreviations

6-4PP	6-4 photoproducts
8-oxodGuo	8-oxo-7,8-dihydro-2'-deoxyguanosine
cDNA	Complementary DNA
CFA	Colony formation ability
CFU	Colony forming units
CPD	Cyclobutane pyrimidine dimers
Cy3	5-Amino-propargyl-2'-deoxyuridine 5'-triphosphate coupled to Cy3 fluorescent dye (Cy3-AP3-dUTP); 5-Amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy3 fluorescent dye (Cy3-AP3-dCTP)
Cy5	5-Amino-propargyl-2'-deoxyuridine 5'-triphosphate coupled to Cy5 fluorescent dye (Cy5-AP3-dUTP); 5-Amino-propargyl-2'-deoxycytidine 5'-triphosphate coupled to Cy3.5 fluorescent dye (Cy3.5-AP3-dCTP)
DLR-ME	German Aerospace Center (DLR), Institute of Aerospace Medicine, Cologne, Germany
DNA	Deoxyribonucleic acid
DPA	2,6-pyridine-dicarboxylic acid (dipicolinic acid)
DSMZ	German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany
GCR	Galactic cosmic rays
GO	Gene ontology
IC	Inactivation constant
HIMAC	Heavy Ions Medical Accelerator at NIRS, Inage-Chiba, Japan
HR	Homologous recombination
HZE particles	High-energy charged particles
HZI	Helmholtz Centre for Infection Research at Braunschweig, Germany
LET	Linear energy transfer
MPI-IB	Max Planck Institute of Infection Biology at Berlin, Germany
NER	Nucleotide excision repair

NHEJ	Non-homologous end-joining
NIRS	National Institute of Radiological Sciences, Inage-Chiba, Japan
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction(s)
φ-facilities	Space and Planetary Simulation Facilities at DLR, Cologne, Germany
PP	UV-induced DNA photoproducts
rpm	Round(s) per minute
mRNA	Messenger RNA (ribonucleic acid)
rRNA	Ribosomal RNA
RR	Recombination-mediated DNA repair
RT-PCR	Reverse transcriptase-PCR
σ-factor	Sigma factor
ShP	Shock pressure (in GPa)
SASPs	Small, acid-soluble spore proteins
SDS	Sodium dodecyl sulphate
SP	Spore photoproduct
TDHT	5,6-dihydro-5(α-thyminyl)thymine (= spore photoproduct)
ThdGly	Thymidine glycols or thymidine glycol adducts
UV	Ultraviolet radiation (in J/m^2)
X-rays	X-ray-irradiation (in Gy)
v/v	volume/volume
w/v	weight/volume
v/w	volume/weight

1. Introduction

1.1 Bacterial endospores stretch the limits of life

Bacterial endospores have since been recognized as the hardiest known form of life on Earth, and considerable effort has been invested in understanding the molecular mechanisms responsible for the almost unbelievable resistance of spores to environments which exist at (and beyond) the physical extremes which can support terrestrial life (Nicholson et al., 2000). Examples of sporeforming bacteria are rather widespread within the low G+C subdivision of gram-positive bacteria. They represent inhabitants of diverse habitats, such as aerobic heterotrophs (*Bacillus* and *Sporosarcina* sp.), halophilic (*Sporosarcina halophila* and *Sporohalobacter* sp. as a rare gram-negative representative), microaerophilic lactate fermenters (*Sporolactobacillus* sp.), anaerobes (*Clostridium* and *Anaerobacter* sp.), sulfate reducers *(Desulfotomaculum* sp.), and even phototrophs (*Heliobacterium* and *Heliophilum* sp.). The most detailed molecular information of sporeforming bacteria has been obtained from studies of species belonging to the genera *Bacillus* and *Clostridium*. *Bacillus* is a genus of rod-shaped, sporeforming, gram-positive bacteria and a member of the division *Firmicutes* (kingdom *Bacteria*). *Bacillus* species are either obligate or facultative aerobes, and tested positive for the enzyme catalase. These characteristics originally defined the genus, but not all such species are closely related, and many have been moved to other genera (e.g. *Paenibacillus*, *Geobacillus*, *Salibacillus*).

Endospores (later on called spores for convenience) of *Bacillus* sp. are ubiquitous and can be isolated in almost every niche in nature and they are relocated spatially via wind, water, living hosts, etc., to environments potentially favorable for germination and resumption of vegetative growth. As a result, *Bacillus* species and their endospores can be found in environmental samples obtained from all parts of the Earth, both above (bacterial spores were collected at high altitudes up to 77 km) (Imshenetsky et al., 1977) and below (spores of *Bacillus infernus* were isolated from ca. 2700 m below the land surface) (Boone et al., 1995) the surface, and as such represent a highly successful strategy for the survival and widespread dispersal of microbial life. Dormant spores exhibit incredible longevity; reliable reports exist of the recovery and revival of spores from environmental samples (i.e. lake sediments) as old as 10^5 years (Cano et al., 1994; Nicholson et al., 2000). A (still controversially discussed) publication reports about the recovery of viable *Bacillus sphaericus* spores from the gut of a bee fossilized in

Dominican amber with an estimated age of 25 - 40 million years (Cano and Borucki, 1995); furthermore, the isolation of a 250 million-year-old halotolerant spore-forming bacteria (based on its 16S rDNA the microorganismus is a part of the lineage of *Bacillus marismortui* and *Virgibacillus pantothenticus*) from a primary salt crystal has been reported (Vreeland et al., 2000). Because of their high resistance to environmental extremes and their reported longevity bacterial spores have also been suggested as potential candidates for "*Lithopanspermia*", the hypothetical transfer of (microbial) life between the planets of our Solar System via meteorites (Hoch and Losick, 1997; Fajardo-Cavazos et al., 2005).

Under stressful environmental conditions, bacterial cells may produce endospores that can stay dormant for extended periods. Every time during sporulation cells have to "prepare for the worst" and they invest a lot of energy and time to form this unique form in microbial differentiation, a spore. However, longevity of spores can not be predicted, it largely depends on the environment conditions a spore is confronted when in the dormant state.

In the soil environment where many sporeforming bacteria are found, such potentially lethal extreme conditions can include cycles of heat and cold, freezing-thawing, physical abrasion, extreme desiccation, exposure corrosive chemicals, attack by other organisms and their extracellular degradative enzymes, and prolonged exposure to solar radiation (Horneck et al., 1984; Nicholson, 2002; 2004; Nicholson et al., 2005). To survive such stressful conditions, spores must either prevent damage, which would inactivate critical cellular components needed for successful germination and resumption of growth or repair or replace those damaged critical components during spore germination, before their inactivation results in cell death.

1.2 Impact of sporulation on the life cycle of *Bacillus subtilis*

Endospore formation in *B. subtilis* is one often used model systems for studying cellular differentiation because of the relative simplicity of the organism and its amenability to the tools of traditional and molecular genetics (Piggot and Losick, 2002; Hilbert and Piggot, 2004). The differentiation of each cell type is governed by its own program of gene expression and it is coordinated with the differentiation of other cells by intercellular signaling pathways. Endospore/spore formation is a seven to eight hour

process that is triggered by conditions of nutrient limitation (Nicholson et al., 2000). Spore formation involves three cell types known as the predivisional cell, the forespore ("prespore") and the mother cell. Cells enter the pathway to sporulate in response to conditions of mainly nutrient limitation, which results in the formation of the predivisional cell. The predivisional cell undergoes a process of asymmetric division in which a septum is formed near a pole, thereby creating dissimilar-sized progeny cells (Wang et al., 2006). These are the forespore and the mother cell. Initially, the forespore and the mother cell lie side-by-side but later in development the forespore is engulfed by the mother cell in a phagocytic-like process. During subsequent morphogenesis, the forespore, which becomes the core of the mature spore, undergoes dehydration driven in part by the replacement of water by calcium dipicolinate, and its chromosome becomes packaged by a family of small, acid-soluble proteins (SAPSs) into a toroid-like structure in which it is protected against many types of DNA damage (Setlow, 1995). When morphogenesis is complete the spore core is encased by protective outer layers of cortex and coat material. The spore is released by lysis of the mother cell. The master regulator for entry into sporulation is Spo0A, a member of the response regulator family of DNA-binding proteins (Hoch, 1993; Stephenson and Hoch, 2002). Spo0A, which is activated by phosphorylation, orchestrates gene expression in the predivisional cell, acting both as an activator and a repressor. Spo0A controls (directly and indirectly) at least 525 genes, representing about 12 % of the annotated protein-coding genes in the *B. subtilis* 168 genome, indicating that an important role of Spo0A is to shut down the expression of many genes that are normally expressed during vegetative growth (Wang et al., 2006). The levels of Spo0A rise gradually in the early stages of sporulation with different genes being turned on or off at different levels of phosphorylated Spo0A (Fujita and Losick, 2005).

Asymmetric division sets in motion two parallel but interconnected programs of gene expression. Endospore formation is a complex developmental process that requires the expression of more than 200 genes (Losick and Stragier, 1992; Stragier and Losick, 1996). During the endospore development, four RNA polymerases (RNAP) sigma factors appear, displacing one another and conferring on RNAP different specificities for the recognition of different classes of promotors. The sigma factors appear in order σ^E, σ^F, σ^G, and σ^K. This process of altering transcriptional specificity is the fundamental mechanism that regulates sporulation gene expression. The sequential appearance of each sigma factor determines the temporal pattern of gene transcription.

Different sets of genes are expressed in the forespore and in the mother cell. The sigma factors also play a critical role in determining cell-type specific patterns of gene expression. σ^F and σ^G are active exclusively in the forespore, while σ^E and σ^K are active exclusively in the mother cell. The σ^G is an activator that turns on a set of essential ("survival") genes in this cascade, which include genes for the SASP family of proteins, the spore photoproduct lyase (SP lyase) and both enzymes of the non-homologous end-joining (NHEJ) mechanism.

In short, the forespore line of gene expression consists of many genes that contribute to spore morphogenesis and to the resistance and germination properties of the spore. In Fig. 1-1 the scheme of the regulatory network of the controlling sigma factors in *B. subtilis* sporulation is shown.

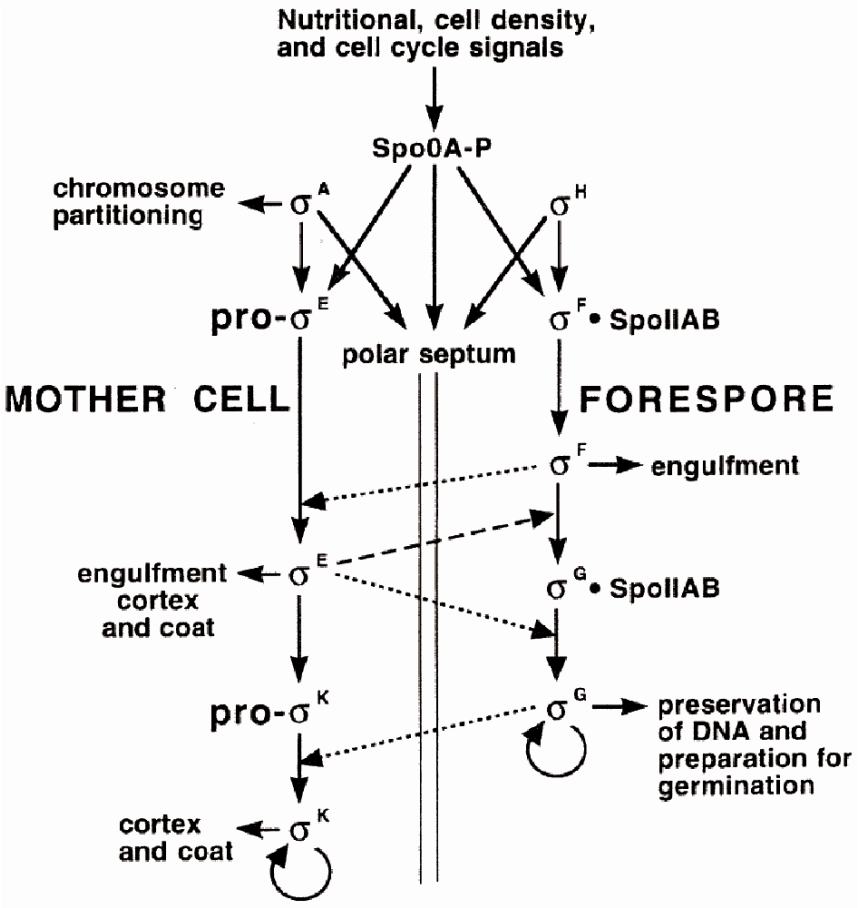


Fig. 1-1 Regulatory network controlling σ factor synthesis and activation. Solid arrows indicate dependence relationships for σ factors and the product of genes that bring about morphological change. The two vertical lines represent the membranes that separate the mother cell and forespore after polar septum formation. Dashed arrows show signaling interactions between the two cell types that govern σ factor activation (short dashes) or synthesis (long dashes), according to Kroos et al. (1999).

Recently, and while this work was in progress, an analysis of genes under control of σ^F and σ^G was reported largely based on transcriptional profiling during sporulation (Steil et al., 2005; Wang et al.,

2006). The mother cell and forespore programs of gene expression consist of about 504 genes, with 383 mother-cell-specific genes and 129 fore spore-specific genes.

The spore chromosome constitutes the only copy of the *B. subtilis* genome during dormancy. Whereas gene expression in the mother cell indirectly contributes to the protection of the genetic material by formation of the spore cortex and the spore structures, the most contributions to spore DNA protection and repair are achieved by the products of genes expressed in the forespore. At least 24 members of the forespore regulon (about 20 % of the forespore-specific genes) are involved. Mainly, small DNA binding proteins (SAPSs) that bind to the spore chromosome, as well as several repair enzymes among others e.g. SP lyase (*splAB*) and NHEJ (*ykoVU*), complete the arsenal of spore-specific proteins (enzymes) involved in maintaining the integrity of the single spore genome copy.

1.3 Factors contributing to spore resistance

Laboratory studies of the resistance of *Bacillus* and *Clostridium* sp. spores to a variety of stress treatments have identified a number of factors that determine the level of spore resistance. They include the genetic makeup and spore morphological attributes e.g. specific features of the spore coats and core (Nicholson et al., 1990; Mohr et al., 1991; Driks, 1999). Their relative significance in spore resistance varies with regard to the phylogenetic origin of the sporeforming species and the kind of stressor (Setlow, 2006). This work deals with the resistance of *B. subtilis* spores. While mutations in a number of individual genes alter specific factors involved in the spore survival (Setlow, 1995; Setlow and Setlow, 1996), it is clear that in the wild-type *B. subtilis* spores the overall genomic information is extremely important in determining levels of spore resistance (Setlow, 1992). In Fig. 1-2 a cross-section image of an endospore of *B. subtilis* is shown. The spore-genome is contained in the nucleoid within the spore core. The core is surrounded by the protective cortex and the lamellar inner spore coat and outer spore coat. The spore core area is 0.2 - 0.3 µm².

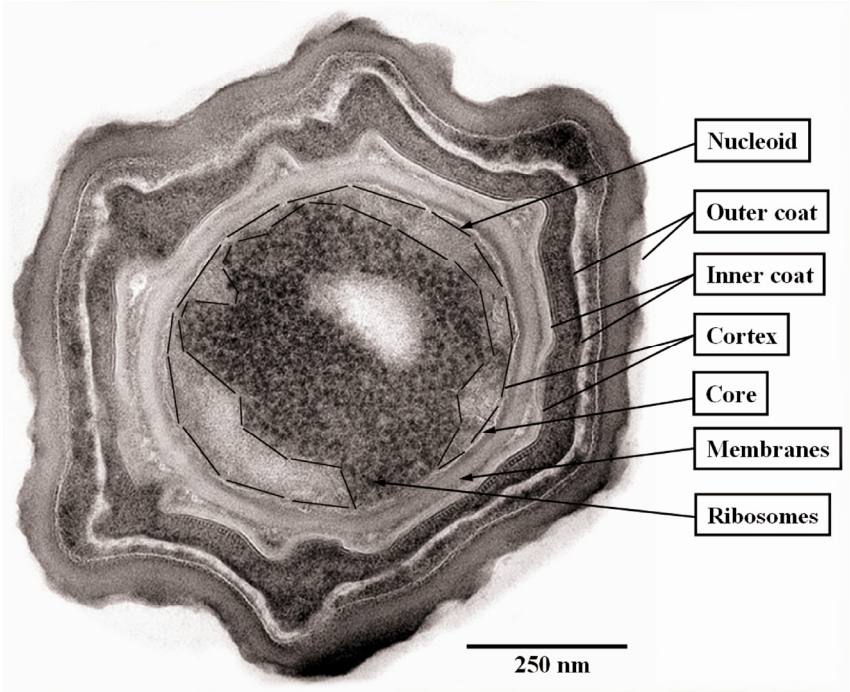


Fig. 1-2 Electron micrograph of a *B. subtilis* 168 (wild-type) spore (modified after Frenkiel-Krispin et al., 2004) after staining with uranyl acetate. The densely stained particles are ribosomes, the ribosome-free spaces in the periphery of the spore core contain chromatin. The sections probed were about 70 nm thick and a segment of a toroidal structure of the nucleoid (bordered view) was detected (Frenkiel-Krispin et al. screened over 3000 cell slices).

Sporulation conditions. Many studies – mainly dealing with wet-heat resistance of spores (Marquis and Shin, 1994), have shown that modulation of the sporulation conditions has a significant effect on the resistance of the spores (Nicholson et al., 2000). Parameters that have been varied include metal ion concentrations and temperature. Whereas the interpretation of changes in spore resistance with variation of the mineral ion content of the sporulation media has been difficult on a mechanistic basis, sporulation at an elevated temperature invariably leads to an increase in the heat resistance (Schaeffer et al., 1965). This effect is mediated by a decrease in core water content during sporulation at higher temperatures. The water content is presumably triggered during late sporulation events (Wang et al., 2006), however the mechanism(s) controlling the content of spore core water is not yet fully understood.

Spore coat layers. The spore coats appear to play some role in spore resistance, especially in preventing the access of peptidoglycan-lytic enzymes to the spore cortex and as barrier for various chemicals e.g. hydrogen peroxide and alkylating agents. Their compositions contribute to the resistance to lysozyme and other bactericidal substances. However, the precise role(s) of individual coat proteins in these resistance properties is still not clear (Riesenman and Nicholson, 2000). Experiments with

spore coat-defective mutants showed the relative contributions of spore coat layers to UV and/or H_2O_2 resistance. Spores of coat-defective strains were similarly resistant to UV-C (254 nm) as wild-type spores, however, they showed a decreased sensitivity to UV (>290 nm) radiation. These observations indicate that the spore coat plays a role in the spore resistance to environmentally relevant UV wavelengths. It was found that pigmented spores show a higher resistance to UV (>320 nm) radiation than no-pigmented ones (Mitchell et al., 1986; Moeller et al., 2005). Spore pigments, such as the carotene neoxanthin, may serve as UV-screening components and/or as antioxidant scavengers of radical oxygen species (ROS) (Edwards et al., 2006).

Spore core. The spore core exhibits relatively low permeability to hydrophilic molecules > 200 Dalton (Da) (Gerhardt et al., 1972). A spore has two membranes; either one or both could be the permeability barrier restricting entry into the spore core. It is not clear if the outer membrane is an intact membrane in the mature dormant spore. But it seems that the outer membrane reacts as a functional membrane (Nicholson et al., 2000). The inner membrane is also an intact membrane and significantly compressed in the dormant spore (Stewart et al., 1980).

In view of the impossibility of DNA repair during (potentially long periods of) dormancy, spores have only two possible methods for coping with deleterious effects of DNA damage in order to maintain their viability: either ensure rapid repair of any DNA damage when spores germinate (i.e. return to vegetative life) before their inactivation results in cell death, and resume metabolism, rapidly accumulate nucleoside triphosphates, and begin macromolecular biosynthesis, and/or (ii) protect dormant spore DNA from damage in the first place. Recent and past studies implicate that there is a synergetic effect of both strategies for ensuring the unique spore resistance and survival (Setlow, 1992; 1995). The most important mechanism for ensuring survival of spore DNA appears to be the prevention of DNA damage in the dormant spore. Major factors involved in preventing DNA damage in dormant spores might well be found in the environment of the spore DNA: i.e. in the spore core. General environmental features as: (**a**) pH, (**b**) water content and (**c**) small molecules are unique to the dormant spore core:

(**a**) The pH in the core of the dormant spore is 6.5 - 7.0 and is approximately one unit lower than the value in growing cells. The decreases in the spore pH originate in the late stage of the sporulation (Magill et al., 1994; 1996) but there is no evidence indicating that the change in pH plays any

significant role in protecting spore DNA (Setlow, 1994). The lower spore pH would actually increase the rate of DNA depurination, an acid catalyzed process (Lindahl and Nyberg, 1972).

(**b**) The water content of the spore core is significantly lower than the value in growing cells. While the water content of the cortex, coat, and exosporium regions of a spore suspended in water is similar to that in growing cells (75-80 % of the wet weight), the water content of the spore core is much lower (28-50 % of wet weight) (Gerhardt and Marquis, 1989). A precise mechanism by which the core's water content is reduced during sporulation is not yet known. This decreased spore-core water content plays a major role in spore resistance to heat and ROS. It should be noted that there is a correlation across *Bacillus* species between lower spore-core water content and increased spore heat resistance. There are no data available, on either the amount of free water (if there is any) in the spore core or the distribution of water among the unique spore-core components. But there is certainly enough water remaining in the spore core to hydrate the spore DNA.

(**c**) Spores have very high levels of divalent ions, in particular Ca^{2+}, with the great majority of those cations being present in the spore core (Murrell, 1967). The amounts and identities of the major cations in spores can be varied, either by alterations in the metal ion content of the sporulation medium (Slepecky and Foster, 1959) or by removal of spore metal ions by titration to low pH and the back-titration to pH 7 with appropriate metal ion hydroxide (Bender and Marquis, 1985). Several metal ions were identified e.g. Na^{+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+}. Marquis and Shin were able to show with spores of *B. stearothermophilus* that an increased spore core mineralization is associated with increased resistance to oxidizing agents (Marquis and Shin, 1994).

A general feature of the environment in the spore core is the presence of an enormous depot of pyridine-2,6-dicarboxylic acid (DPA, dipicolinic acid), which makes up about 10 % of the dry weight of the bacterial spores (Kimura and Sasakawa, 1975; Douki et al., 2005b). DPA is not present in growing cells and is acquired by the developing spores in the late sporulation stage - the time of acquisition of spore heat resistance - in an enzymatic reaction: diketopimelic acid and ammonia react to dipicolinic acid (Kimura and Sasakawa, 1975), DPA exists in spores as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (Setlow, 1994). Studies in *B. subtilis* have shown that spores lacking DPA due to a specific mutation in the *spoVFA* or *spoVFB* locus (also called *dpaA* and *dpaB*), which encode the two subunits of DPA synthetase (Daniel and Errington, 1993), have significantly increased spore

water content and decreased heat and H_2O_2 resistance (Balassa et al., 1979a; 1979b). DPA-less spores exhibit no decrease in UV resistance and are actually more UV resistant than their isogenic wild-type counterparts. This is not unexpected, as DPA has been shown to be a photosensitizer in spores (Setlow, 1995). Spores of *B. cereus* that lack DPA are more heat sensitive than their wild-type spores, but DPA is not essential for the full spore heat resistance. Other factors e.g. spore water content are also related to the spore heat resistance (Murrell and Scott, 1966).

Spore DNA topology and DNA-binding proteins. Spores of *B. subtilis* appear to be monogenomic in their chromosome; thus, there is no protection provided by duplication of genetic information (Nicholson et al., 2005). Spore DNA is saturated with a group of unique proteins called α/β-type small, acid-soluble spore proteins (SASP) (Nicholson et al., 1990). These small proteins (60-75 %) are synthesized only during sporulation (3 - 6 % of the total spore proteins) in the developing spore and are degraded to amino acids in the first minutes of spore germination (Setlow, 2006). One SASP-specific protease (*gpr*, a spore protease) has been identified in germinating spores of *B. subtilis* (Paidhungat and Setlow, 2000; Ragkousi et al., 2000). The SASP proteins bind to DNA largely on the outside of the DNA while changing the DNA from the B-like to the A-like helix conformation (Mohr et al., 1991; Setlow et al., 1992). DNA properties and reactivity to a variety of physicochemical treatments are dramatically changed when DNA is bound by α/β-type SASP. Spores deficient in their α/β-type SASP formation are much more sensitive to UV radiation, heat oxidizing agents, and freeze-drying than the wild-type spores.

The saturation of spore DNA with α/β-type SASP is the major cause of the altered UV photochemistry of DNA in dormant spores. Binding of α/β-type SASP protects spore DNA from attack by several types of toxic chemicals (e.g. peroxides). However, α/β-type SASP binding plays no role in DNA protection against alkylating agents, although these chemicals cause damage to the DNA (Setlow et al., 1998). So far, 16 genes have been identified to encode for major or minor small, acid-soluble spore proteins. With the exception of *sspA* (major α-type SASP) and *sspB* (major β-type SASP), little work has been performed on the function of the other SASP proteins.

Summary of the role of the spore components in spore resistance. All spore-specific factors mentioned above are important in at least one or more spore resistance property. Their importance varies considerably both for the same resistance in spores of different species (e.g. *Clostridium* and *Bacillus*) and for different resistance properties within the same species. Table 1-1 summarizes the involvement of the various factors in spore resistance of *Bacillus* species to different treatments (from Setlow, 1995; 2006; Nicholson et al., 2000; 2005).

Table 1-1 Role of various factors in the spore resistance of *Bacillus* species to different treatments

	Effect on spore resistance				
Treatment	SASP	Core water	Core minerals	Spore coats	Sporulation conditions
Desiccation	+	–	?	?	?
Dry heat	+	–	+	?	?
Wet heat	+	+	+	?	+
Ionizing radiation	–/+[a] (?)	–/+[a] (?)	?	?	?
UV radiation	+	–	?	–/+[b]	–
Glutaraldehyde	–	?	?	+	?
Peroxides	+	+	?	+	?
Alkylating agents	+	?	?	–	?

Symbols: + importance; – no effect; –/+ some importance but has some effect; ? no data available.
[a]Low spore core water content (Nicholson et al., 2000) and DNA protecting by SASP binding (Setlow, 1995) are likely important in ionizing radiation resistance, but that had not been shown by experiment. The role of other spore components in ionizing radiation resistance has not been systematically evaluated. [b]Spore coats play a minor role in resistance to UV-B and UV-A wavelengths (Riesenman and Nicholson, 2000).

1.4 Role of germination in spore's survival

In the spore state, DNA damage may accumulate, but it can only be repaired or restored after the initiation of germination, which is the first crucial step in the return of spores to vegetative growth. Spore germination begins with the addition of a compound that causes the initiation of germination (a germinant, e.g. single/or a combination of amino acids, sugars) or a variety of non-nutrient agents (e.g. heat and pressure) (Moir and Smith, 1990) and continues through a number of irreversible reactions (loss of specific resistance properties); this process does not require metabolic energy (Fig. 1-3). Spore

germination is divided into two stages. In stage I nutrient germinants bind to receptors in the spore's inner membrane and this interaction triggers the release of the spore core components e.g. dipicolinic acid and cations, and replacement of these components by water (hydration). It takes only seconds up to minutes before some of these events e.g. ion and DPA release and partial core rehydration are initiated. In stage II the hydrolysis of the spore peptidoglycan cortex and the subsequent germ cell wall expansion allow the complete full spore core hydration and resumption of spore metabolism and macromolecular synthesis (Setlow, 2003). Small acid-soluble protein degradation proceeds only slowly without cortex hydrolysis, but cortex hydrolysis can proceed without SASP hydrolysis. SASP and cortex degradation take at least 5 min or more in an individual germinating spore.

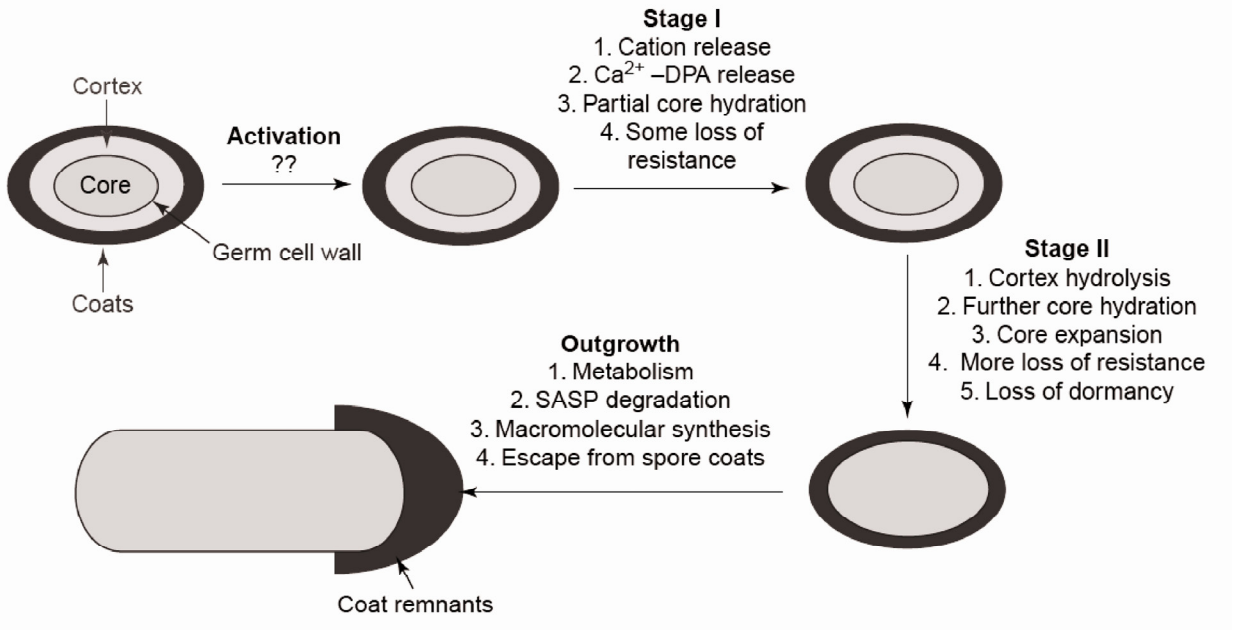


Fig. 1-3 Events in spore germination stage I and II and in outgrowth (Setlow, 2003).

In contrast, outgrowth, which follows germination and continues until conversion back into a vegetative cell, generally at the first cell division, requires both metabolism and macromolecular synthesis. The time for completion of outgrowth is dependent on the medium and it takes 60 to 100 min for *B. subtilis* (Setlow, 2003). Several mechanisms during spore germination e.g. activation, commitment of germinants and initial steps (such as DNA repair) in spore germination are poorly understood. Furthermore, within one spore population, some spores germinate readily and some do not (these spores were often called superdormant spores) (Setlow, 2003).

1.5 DNA repair systems

Despite the protection mechanisms provided by the components of the dormant spore, potentially lethal or mutagenic damage may accumulate in the spore DNA. It is finally the speed and accuracy with which spore DNA damage can be repaired during germination that determines the degree of spore resistance to extreme environments. In Fig. 1-4 an overview of the different types of DNA damaging agents and treatments, their causing lesion and predicted DNA repair pathways is shown (according to Fleck and Nielsen, 2004).

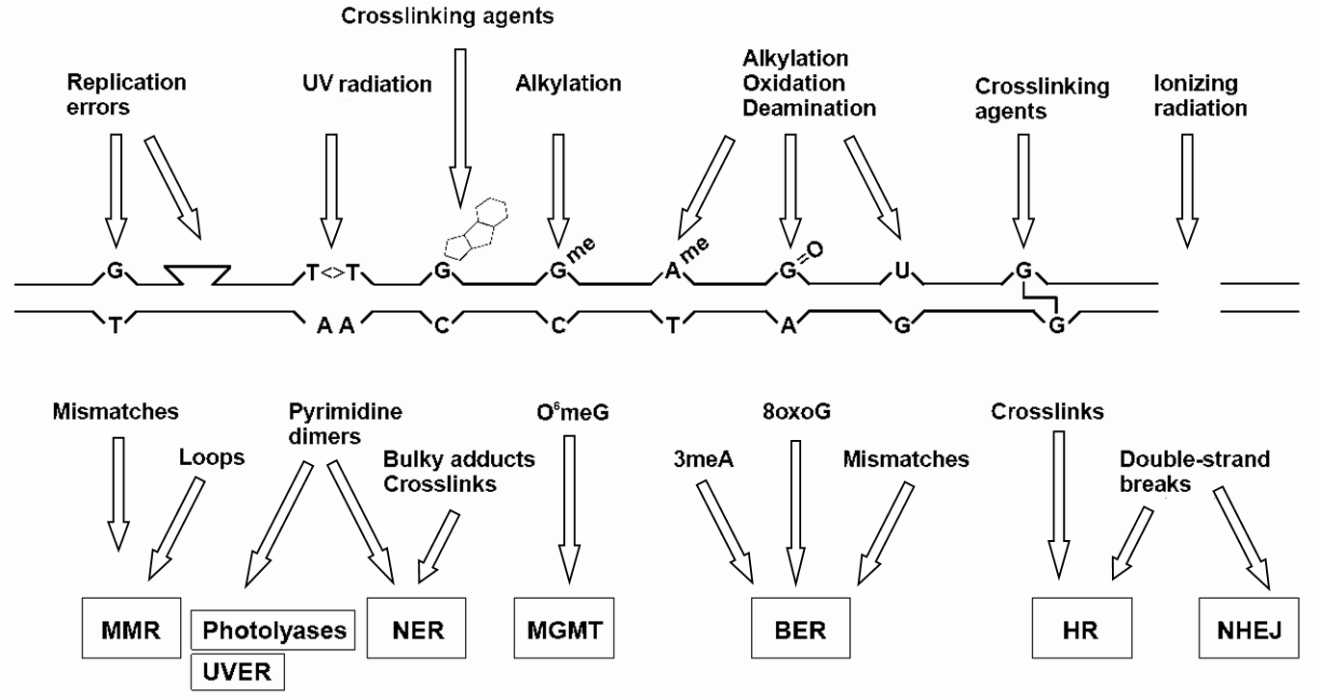


Fig. 1-4 Summary of different types of DNA damaging agents and treatments, their induced lesion and predicted DNA repair pathway (adapted from Fleck and Nielsen, 2004). Used abbreviations in this overview: MMR mismatch repair, UVER UV endonuclease, NER nucleotide excision repair, O^6meG O^6-methylguanine, MGMT O^6-methylguanine-DNA-methyltransferase, 3meA 3-methyladenine, 8oxoG 8-oxo-7,8-dihydroguanine, BER base excision repair, HR homologous recombination, NHEJ non-homologous end-joining.

Laboratory studies have shown that the critical time for DNA repair during germination is quite short e.g. in germinating spores of *B. megaterium* the *de novo* RNA and protein synthesis begins within the first 5 min of germination, using entirely endogenous reserves of precursors and energy (Setlow and Primus, 1975). Abasic sites, helix-distorting lesions such as the UV-induced spore photoproduct (SP),

or breaks in the phosphodiester backbone of spore DNA could exert potentially lethal effects early in germination by blocking the progress of RNA polymerase, thus halting expression on any number of critical pathways leading to replicative DNA synthesis and outgrowth. In addition, unrepaired lesions in spore DNA itself would physically block replication, leading to both lethal and mutagenic consequences (Friedberg et al., 1995). A complete catalog of all types of damage incurred in DNA of spores exposed to extreme environments as well as the involved DNA repair systems during germination was not yet obtained e.g. relatively little work on the role of specific DNA repair systems or on the nature of the DNA damage caused by ionizing radiation of spores has been performed (Setlow, 1995; 2006; Nicholson et al., 2000).

Lesions can be removed from damaged DNA through the action of such systems as nucleotide excision repair (NER), spore photoproduct lyase (SP lyase), base excision repair (BER), adaptive response, and oxidative stress response (Sancar, 1996). Bulky noncoding lesions in the DNA that are not removed before being encountered by the replication machinery constitute a block to further DNA synthesis. The blockage in DNA replication can be circumvented if the DNA polymerase dissociates from the DNA upon encountering a noncoding lesion and then reinitiates replication on the other side of the lesion (Rupp and Howard-Flanders, 1968). The resulting gaps in the newly synthesized daughter strand can then be "repaired" by process called recombinational repair (recombination-mediated DNA repair) (Walker, 1984). This type of mechanism exemplifies tolerance of DNA damage rather than a typical DNA repair process since the actual damage is never physiologically removed but is diluted out by subsequent DNA replication.

DNA repair systems were considered integral parts of an organism's ability to survive the effects of environmental insults and metabolic processes:

Photoreactivation. This DNA repair mechanism reduces the deleterious effects of UV irradiation (200 - 300 nm) by means of a (VIS)radiation-dependent process in which the *cis-syn* cyclobutyl pyrimidine dimers are enzymatically monomerized by a photolyase (Friedberg, 1985). Interestingly, *B. subtilis* (and several other *Bacillus* and *Clostridium* species) lacks this type of DNA repair system (Yasbin, 1985).

Nucleotide excision repair. Bulky, noncoding lesions, e.g. UV-induced photoproducts (alteration of the nucleotide structure), that produce a block to DNA replication can be removed from damaged DNA through the action of a nucleotide excision repair (NER) system. This repair system in *B. subtilis* closely resembles the analogous system in *E. coli*, which has been extremely well characterized (Lin and Sancar, 1990, 1992; Friedberg, 1996). The molecular details of NER in *B. subtilis* have not been elucidated to the same degree as NER in *E. coli*. The *B. subtilis* homologs of the genes encoding the UvrA, UvrB and UvrC subunits of the *E. coli* excinuclease have been identified, mapped, cloned and sequenced (Chen et al., 1989; Kunst et al., 1997). Regulation of expression of some genes for the *B. subtilis* NER pathway has been studied using *uvr-lacZ* fusions (Cheo et al., 1991). In this NER system, a variety of bulky lesions are recognized and endonucleolytic cleavage of the DNA is initiated, both 3' and 5' to the lesion, by the activities of the UvrA, UvrB and UvrC proteins causing single strand nicks on either sides of the UV-induced dimers (Kushner, 1987). Furthermore, DNA helicase and DNA polymerase I play also critical, but not essential, roles in the further processing of lesions and the resynthesis (e.g. gap filling) of the DNA (Visse et al., 1991). Essentially, following UV irradiation (or treatments with other DNA-damaging treatments) lesions are enzymatically removed from the DNA. Expression of the *uvr*-system is inducible by DNA damage both during vegetative growth (Cheo et al., 1991) and during the outgrowth phase of germination of UV-C irradiated spores (Setlow and Setlow, 1996).

Base excision repair. Base excision repair (BER), another pathway to remove noncoding lesions from DNA, differs from NER in that damaged or incorrect bases are excised as free bases rather than nucleotides or oligonucleotides. Total removal of the DNA lesion requires a two-step process. First, there is hydrolysis of the *N*-glycosylic bond that links the inappropriate base or the lesion to the deoxyribose-phosphate backbone of DNA by a repair enzyme(s) called DNA gylycosylase(s). Once a damaged base or incorrect base is recognized by a specific glycosylase, the *N*-glycosylic bond is cut, leaving an apurinic or apyrimidinic (AP) site in the DNA (Lindahl, 1982). The second step in BER is removal of this AP site via the action of one or more nucleases. Abasic sites in DNA are specifically recognized by enzymes known as AP endonucleases. Repair synthesis and ligation in BER proceed as in NER. BER mechanisms have been identified in *B. subtilis*, the involvement of glycosylase in this

mechanisms suggest that this enzyme is a base-specific excision repair protein (Radany et al., 1997a; 1997b).

A spore-specific DNA repair pathway, the SP lyase. The spores of *B. subtilis* and other *Bacillus* species are much more resistant (up to 50-fold) to the effects of UV irradiation than are their growing vegetative cells. Upon spore germination, UV resistance increases to even higher levels before declining to the level of resistance of exponential and stationary growth phase vegetative cells. Early studies of the UV photochemistry of DNA in spores of *Bacillus* species led to the discovery that the *cis-syn* cyclobutane-type pyrimidine dimers, of which the thymine-thymine (TT) dimers represents the primary photoproduct found in the DNA of UV irradiated vegetative cells, was not induced in the DNA of UV-irradiated spores (Donnellan Jr. and Setlow, 1965; Varghese, 1970). In contrast to vegetative cells, a different series of UV photoproducts was identified within the DNA of spores (Lindberg and Horneck, 1992; Douki et al., 2005a; 2005b). The primary photoproduct of UV-irradiated spores is 5,6-dihydro-5(α-thyminyl)thymine (THDT), known as the "spore photoproduct", SP (Varghese, 1970). Unlike the generalized NER system and the RecA-mediated (Rec) pathway (see below), which operate in both vegetative cells and outgrowing spores on a variety of types of DNA damage (Setlow, 1995; Setlow and Setlow, 1996; Wang et al., 2006), spore photoproduct lyase (SP lyase) is specifically dedicated to the repair of the spore photoproduct (SP) which has accumulated in dormant spores exposed to UV radiation. SP lyase is produced upon transcription of *splB* by sigma-G RNA polymerase at morphological stage III in the developing forespore, where the enzyme is packed in the dormant spore; it is activated during germination (Pedraza-Reyes et al., 1994; 1997; Fajardo-Cavazos and Nicholson, 2000). Transcription of *splB* is not observed during either vegetative growth or germination, nor is it induced by DNA damage during either vegetative growth or germination (Pedraza-Reyes et al., 1994; Au et al., 2005). SP lyase is activated during early germination to monomerize the SP dimer back to the two original thymine residues in an adenosyl-radical dependent (“Radical SAM”) reaction (Munakata and Rupert, 1972; 1974; 1975; Nicholson et al., 1997; Slieman et al., 2000; Rebeil and Nicholson, 2001). From the nucleotide sequence of the region, it was observed that the SP lyase (*spl*) locus was organized as a bicistronic operon, consisting of *splA*, encoding a protein of 79 amino acids (aa) and 9.2 kDa of unknown function, and *splB*, encoding a 40-kDa protein which exhibited limited regional homology to the DNA photolyase/6-4 photolyase/blue-light photoreceptor family of proteins

(Fajardo-Cavazos et al., 1993; Nicholson and Fajardo-Cavazos, 1997). The deduced SplA amino acid sequence is similar to that of another small regulatory protein, the *trp* RNA-binding attenuation protein (TRAP) of *B. subtilis*. SplA acts mainly as the transcriptional negative regulator of the SP lyase operon (Fajardo-Cavazos and Nicholson, 1995; Rebeil et al., 1998). Interestingly, genetic and biochemical experiments performed in the late 1990 indicated that SP lyase is a member of the S-adenosyl-methionine (SAM)-dependent, radical utilizing enzyme family and is a unique DNA repair protein which utilizes a radical mechanism by splitting the SAM complex to generate methionine and a 5'-adenosyl radical for (further) direct and indirect catalytic reaction(s) (Rebeil et al., 1998). The reaction likely proceeds by formation of a 5'-adenosyl radical that abstracts a proton from SP generating an SP radical (Cheek and Broderick, 2002). Formation of this radical then leads to β-scission of the thyminyl-thymine bond and finally completion of the reaction by transfer of a proton from 5'-adenosine back to thymine thus recycling the 5'-adenosyl radical (Mehl and Begley, 1999).

SOS response of *B. subtilis*. The regulation of the SOS (a postreplication DNA repair system activates the DNA replication to bypass lesions or errors in the DNA) system that controls the expression of a number of DNA repair genes has been well studied in *E. coli* (Friedberg et al., 1995; Courcelle et al., 2001); the SOS response of *B. subtilis* appears to be generally similar (Yasbin et al., 1993; Au et al., 2005). In *B. subtilis*, RecA is an initial sensor of DNA damage, which can be generated in cells by a variety of treatments, including UV, hydrogen peroxide, and cross-linking agents (Yasbin et al., 1993). The DNA damage caused by these treatments may not be sensed directly, but single-stranded DNA regions that arise upon either replication or repair of damaged DNA appear to be sensed in some fashion (Lovett et al., 1993). However, it is possible that different types of DNA damage may be sensed differently. Whatever generates the inducing signal, this signal causes the conversion of RecA into an activated form that stimulates the digestion (probably autodigestion) of the LexA protein (Friedberg et al., 1995; Winterling et al., 1997; 1998). LexA is probably a repressor of many damage repair genes, including *recA* and *lexA* (as well as several other genes; Au et al., 2005) itself, and LexA autodigestion inactivates its repressor function. Consequently, DNA damage results in increased expression of many DNA repair genes, this expression is eventually shut down as DNA damage is repaired, activated RecA is lost, and functional LexA levels rise. Given what is known about this system in *E. coli* and *B. subtilis*, it is not surprising that treatments causing significant spore DNA

damage result in induction of *lexA*- and *recA*-genes during spore outgrowth. Presumably the DNA damage is sensed by RecA during outgrowth, resulting in activation of RecA, cleavage of LexA, relief of repression of the *lexA* and *recA* genes. By analogy with the *E. coli* SOS system, it is likely that the different degrees of induction of these DNA repair genes are due to differences in the affinity of LexA for the control regions of these genes. Various authors reported that *B. subtilis* spores of a *recA* single mutation were no more sensitive to UV-C radiation, wet heat, hydrogen peroxide, formaldehyde and freeze drying than the wild-type spores (Munakata and Rupert, 1975; Setlow and Setlow, 1996; Setlow, 2006), whereas they were significantly more sensitive to dry heat and nitrous acid (Setlow, 2006) compared to their wild-type counterparts. Baltschukat and Horneck (1991) were able to show differences in the survival of *recA* and wild-type spores exposed to high doses of ionizing radiation (X-rays and accelerated heavy ions), but they used in their studies a *B. subtilis* strain carrying a triple mutation i.e. deficient in NER (*uvrB*), SP lyase (*splB*) and in its major recombinogenic protein RecA. So, little is known about the role of recombination-mediated DNA repair of (germinating and outgrowing) spores after various treatments, such as ionizing radiation and extreme desiccation.

Adaptive response. An inducible repair system that protects against the lethal and mutagenic effects of alkylation damage (caused by chemical agents such as methyl methane sulfonate) has been detected in a variety of different organisms. Experiments with alkylating agents in cultures showed an increase in the production of proteins that are capable of accepting methyl groups from the DNA or are capable of removing damaged bases from DNA (Lindahl et al., 1988). In *E. coli* the *ada* gene possesses two distinct DNA methyltransferase activities and it is also the transcriptional activator of the adaptive response (Shevell et al., 1990). O^6-methylguanine-DNA methyltransferase is one of the major enzymes involved in the adaptive response to alkylating agents. This protein was designated as the methylphosphodiester-DNA methyltransferase (Morohoshi and Munakata, 1987). Interestingly, the separate functions of the AdaA and AdaB proteins of *B. subtilis* are handled by a single Ada protein of *E. coli* (Shevell et al., 1990). But these data were obtained for experiments with treated vegetative cells and not with dormant spores; therefore few knowledge has been gathered on this topic.

Oxidative stress response. The enzymes necessary to allow cells to tolerate oxidative stress can be classified in two functional classes: (i) protection enzymes (which remove the harmful agents before

they disrupt essential cell functions) e.g. superoxide dismutase and catalase, which prevent damage caused by reactive oxygen species and (ii) enzymes which actually remove deleterious lesions in cellular components, alkylhydroperoxidase and glutathione reductase are examples of this type of repair action. Three catalase enzymes are known to be produced in *B. subtilis* 168 (Kunst et al., 1997). Bagyan et al. reported that KatA, the major catalase in growing cells of *B. subtilis*, is not present in spores, *katA* is not transcribed until at least 20 min after the initiation of spore germination. Thus, KatX appears to be the only catalase that can detoxify hydrogen peroxide early in spore germination. Presumably, after synthesis of KatA, outgrowing spores of a *katX* mutant will become hydrogen peroxide resistant, but this presumption has not been tested directly (Bagyan et al., 1998).

Other DNA repair systems. In the 1997 sequenced genome of *B. subtilis* 168 (Kunst et al., 1997), a number of open reading frames presumably encoding components of various DNA repair systems have been identified e.g. recently, Weller et al. (2002) identified in vegetative cells of *B. subtilis* bacterial Ku homologs (*ykoV*), which retain the biochemical characteristics of the eukaryotic Ku heterodimer. The bacterial Ku specifically recruits DNA ligase (*ykoU*) to DNA ends and thereby stimulates DNA ligation. Loss of these proteins leads to hypersensitivity of stationary-phase *B. subtilis* cells to ionizing radiation (Weller et al., 2002). Wang et al. reported that the *ykoVU* operon is forespore-expressed and both proteins (*ykoU* and *ykoV*) are active during germination (Wang et al., 2006), but only few information are available on the potential (environmental) inducers, which leading to an activation of the non-homologous end-joining (NHEJ) repair pathway during germination. Furthermore, various base excision repair and mismatch repair systems were also detected (e.g. the recently described YtkD-MutT complex, which is mainly involved in the oxidative stress response of vegetative *B. subtilis* cells; Castellanos-Juàrez et al., 2006). For these additional "new" DNA repair systems almost nothing is known on the role in the spore resistance to extreme environmental conditions.

With the complete genomic sequence of *B. subtilis* 168 in hand (Kunst et al., 1997) began the first attempts at elucidating the complete complement of *B. subtilis* DNA repair genes and their functions. A list of about 60 putative DNA repair/recombination genes was compiled (Sonenshein et al., 2002), about half of which consisted of previously unknown genes identified on the basis of automated sequence homology searches. Only a few of these putative genes (*yqfS, ytkD*) have since been

confirmed by genetic and biochemical attempts aimed at encoding DNA repair enzymes (Salas-Pacheco et al., 2003; Urtiz-Estrada et al., 2003; Ramirez et al., 2004). A recent alternative approach to identifying regulons of bacterial genes whose transcription responds to environmental stressors has been the use of transcriptional microarrays. To date, global microarray-based searches of genes induced by several potential DNA damaging agents (e.g. hydrogen peroxide, UV radiation) have been reported for the prokaryotes *Deinococcus radiodurans* (Liu et al., 2003), *Escherichia coli* (Courcelle et al., 2001; Wei et al., 2001; Zheng et al., 2001), *B. subtilis* (vegetative cells) (Helmann et al., 2003; Au et al., 2005; Goranov et al., 2006), and *Halobacterium* sp. (Baliga et al., 2004), but to date no studies have been reported addressing the global transcriptional response of *B. subtilis* spores to DNA damage after ionizing or mono- and polychromatic UV irradiation or extreme desiccation.

1.6 Aim of this thesis

The extremely high resistance of bacterial endospores to environmental stress factors has intrigued researchers since long time and many characteristic spore features, especially those involved in the protection of spore DNA, have already been uncovered. The disclosure of the complete genomic sequence of *B. subtilis* 168 (Kunst et al., 1997) and the rapid development of transcriptional microarray techniques have opened new opportunities of gaining further insights in the enigma of spore resistance.
It is the aim of this thesis to further elucidate the mechanisms of bacterial endospores resistance by applying those advanced biochemical, genetic and molecular biology techniques. Emphasis has been laid on reaching a better understanding of the DNA protection and repair strategies, which allow *B. subtilis* spores to cope with DNA damage induced by external stress factors. The following physical stress factors of environmental importance - either on Earth or in space - were selected for this thesis: (i) UV radiation, i.e. UV-C (λ = 254 nm) and polychromatic UV-A (λ = 320 - 400 nm); (ii) ionizing radiation, i.e. X-rays and accelerated heavy ions, (iii) exposure to extreme dryness via high vacuum; and (iv) high shock pressures simulating meteorite impacts. To reach a most comprehensive understanding of spore resistance to those harsh terrestrial or simulated extraterrestrial conditions, a standardized experimental protocol of the preparation and analyzing methods was established including the determination of the following spore responses: (i) survival (based on CFA), (ii) induced mutations (genetic specificity by use of PCR or phenotypic morphology), (iii) DNA damage (base modifications by use of HPLC-MS/MS), (iv) role of SASPs in spore resistance by use of different SASP deficient

mutants, (v) role of different repair pathways by use of a set of repair deficient mutants (besides those of already known pathways also those for the recently discovered NHEJ system), and (vi) transcriptional responses during spore germination by use of genome-wide transcriptome analyses and confirmation by RT-PCR. From this comprehensive set of data on spore resistance to a variety of environmental stress parameters a model of a "built-in" transcriptional program of bacterial spores in response to DNA damaging treatments to ensure DNA restoration during germination has been developed.

2. Material and methods

2.1 Cultivation of *Bacillus subtilis* and nucleic acids isolation

2.1.1 Media

All chemicals and ingredients (nutrients and salts) are in (w/v).

Each medium, buffers and solutions were autoclaved at 121°C for 30 min.

Growth medium (nutrient broth (NB) medium; after Horneck et al., 2001a; 2001b)

0.8 % nutrient broth (NB) (Difco Detroit, MI, USA)

0.1 % potassium chloride (KCl) (Merck KGaA, Darmstadt, Germany)

0.02 % magnesium sulfate heptahydrate ($MgSO_4 \times 7\ H_2O$) (Merck KGaA, Darmstadt, Germany)

by addition of 2.0 % agar (Becton, Dickinson and Company, Sparks, MD, USA) for solid medium plates (NB medium plates)

Germination medium (2×Luria Bertani broth (LB) medium; after Setlow et al., 2003)

2.0 % peptone tryptone (Becton, Dickinson and Company, Sparks, MD, USA)

2.0 % yeast extract (Becton, Dickinson and Company, Sparks, MD, USA)

1.0 % sodium chloride (NaCl) (Merck KGaA, Darmstadt, Germany)

Phosphate-buffered saline (SAL; after Pogoda de la Vega et al., 2005)

0.7 % di-sodium phosphate (Na_2HPO_4) (Merck KGaA, Darmstadt, Germany)

0.3 % potassium di-hydrogen phosphate (KH_2PO_4) (Merck KGaA, Darmstadt, Germany)

0.4 % sodium chloride (NaCl) (Merck KGaA, Darmstadt, Germany)

and adjusted pH to 7.5

Schaeffer sporulation medium (SSM; after Schaeffer et al., 1965)

1.6 % nutrient broth (NB)

0.2 % potassium chloride (KCl)

0.05 % magnesium sulfate heptahydrate ($MgSO_4 \times 7\ H_2O$)

adding of following sterile component solutions to the cooled (55°C) medium:

1 ml 1M calcium(II)nitrate ($Ca(NO_3)_2$) (Merck KGaA, Darmstadt, Germany)

1 ml 0.1M manganese dichloride tetrahydrate ($MnCl_2 \times 4H_2O$) (Merck KGaA, Darmstadt, Germany)

1 ml 1mM iron(II)sulfate ($FeSO_4$) (Merck KGaA, Darmstadt, Germany)

2 ml 50 % (w/v) *D*(+)-glucose monohydrate (Merck KGaA, Darmstadt, Germany)

2.1.2 *Bacillus subtilis* strains

Following *B. subtilis* strains were used in this work (Table 2-1).

Table 2-1 *B. subtilis* strains used in this work

Strain (original code)	Genotype or phenotype	Source and/or reference
B. atrophaeus[a]	wild-type	DSM 675, DSMZ (Moeller et al., 2005)
B. subtilis 168[a,b]	*trpC2* (wild-type)	DSM 402, DSMZ (Spizizen, 1958)
B. subtilis PS356[c,d]	*trpC2,*Δ*sspA* Δ*sspB*	P. Setlow (Douki et al., 2005a)
B. subtilis WN463[d,e,f]	*trpC2, recA*::*ermC*	W. L. Nicholson (Cheo et al., 1992)
B. subtilis TKJ6324[d,g,h]	*trpC2, splB1*	N. Munakata (Dose and Gill, 1995)
B. subtilis TKJ6312[d,i]	*trpC2,* Δ*splB* Δ*uvrB*	N. Munakata (Makino and Munakata, 1978)
B. subtilis TKJ8431[d,g]	*trpC2,* Δ*recA* Δ*splB* Δ*uvrB*	N. Munakata (pers. communication)
B. subtilis RM01[d, j]	*trpC2, rpoB* (Q469L)	Laboratory stock (this work)
B. subtilis BFS1845[c,d,k,l]	*trpC2, ykoU*::pMUTIN4	A. J. Doherty (Weller et al., 2002)
B. subtilis BFS1846 [c,d,k,l]	*trpC2, ykoV*::pMUTIN4	A. J. Doherty (Weller et al., 2002)
B. subtilis BFS1846 [c,d,k,l]	*trpC2, ykoUV*::pMUTIN4	A. J. Doherty (Weller et al., 2002)

[a]DSMZ, German Collection of Microorganism and Cell Cultures GmbH, Braunschweig, Germany (*B. atrophaeus* DSM 675, formed red-pigmented, until 2000 listed under *B. subtilis*, reclassified by Fritze and Pukall (2001)), [b]*trp* auxotrophy (i.e. need at least 50 µg tryptophan per ml of minimal media to grow), [c]PS356 form only about 20 % of the wild-type α/β-type SASPs, resistance to chloramphenicol (3 µg/ml), [d]isogenic with strain *B. subtilis* 168, [e]resistance to lincomycin (25 µg/ml) and erythromycin (1 µg/ml), [f]("168 *recA*::*ermC*) containing an insertion of the *ermC* cassette (YB886) into the recA gene, [g]his and met auxotrophy (i.e. need histidine and methionine in minimal media to grow), [h]*splB1* = carrying 2 point mutations causing changes in amino acids G168R and G242D in SP lyase (Fajardo-Cavazos and Nicholson, 1995), [i]his, leu and met auxotrophy (i.e. need histidine, leucine and methionine in minimal media to grow), [j]resistance to rifampicin (50 µg/ml), [k]resistance to erythromycin (3 µg/ml), [l]("168 *ykoUV*::pMUTIN4) containing an insertion of a pMUTIN4 into the respective *ykoU*/*ykoV* gene.

All used amino acids (*L*-alanine, *L*-histidine, *L*-leucine, *L*-methionine, *L*-tryptophan) and tested antibiotics (chloramphenicol, erythromycin, lincomycin, rifampicin) were obtained from Merck KGaA, Darmstadt, Germany and used after their proposed recommendations (i.e. concentration and solvent).

2.1.3 Cultivation of vegetative cells

Vegetative cells of all strains (2.1.2, Table 2-1) were routinely cultured on the recommendation of the DSMZ, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany (http://www.dsmz.de). Cells of *Bacillus* sp. were cultivated under vigorous aeration at 37°C in 5 ml NB medium for 18 h (overnight).

2.1.4 Sporulation, spore purification and storage

Overnight cultures (10 ml) of all used *Bacillus* sp. (as described in 2.1.3) were transferred in 200 ml double-strength liquid SSM medium (Schaeffer et al., 1965) and cultivated under constant aeration for 4-5 d at 37°C until a sporulation frequency of > 95 % had been reached, as judged by phase-contrast microscopy. Sporulated cultures were harvested by centrifugation (10 000*xg*, 20 min, 4°C) and treated with $MgSO_4$ (2.5 µg/ml), lysozyme (200 µg/ml) and DNAse I (2 µg/ml) for 30 min at 37°C in order to destroy the residual vegetative cells. The enzymes were inactivated by heating for 10 min at 80°C. After repeated centrifugation (10 000*xg*, 20 min, 4°C) and washing in distilled water, the purified spores (about 10^{10} spores/ml) were stored in water at 4°C (Baltschukat et al., 1986; Nicholson and Setlow, 1990; Moeller et al., 2005; 2006). All spore preparations used in this work were free (> 98 %) of growing cells, germinating spores and cell debris, as controlled by microscopic observations.

2.1.5 Assay for colony formation ability

For the determination of the total viable cell count of a cell- or spore- suspension, the colony formation ability (CFA) was defined by plating an aliquot on NB medium plates after appropriate dilution in sterile SAL (for vegetative cells, germinating and outgrowing spores) or in sterile water (for dormant spores) and incubated overnight at 37°C. Visible colonies, grown on nutrient agar plates, were counted and multiplied with the used dilution (Horneck et al., 2001a; 2001b; Moeller et al., 2005).

2.1.6 Spore germination

To study DNA processes during spore germination (treated or non-treated) *Bacillus* sp. endospores were transferred into 100 ml 2xLB medium (20.0 g Bacto-Tryptone, 10.0 g Bacto-Yeast extract (BD Diagnostics, Sparks, MD, USA), 10.0 g NaCl (Merck KGaA, Darmstadt, Germany)) with an addition of 4 mM *L*-alanine (Merck KGaA, Darmstadt, Germany) to stimulate initiation of spore germination (Setlow et al., 2001), and incubated at 37°C under vigorous shaking at 300 rpm (described in detail in Woese et al., 1958; Paidhungat and Setlow, 1999; Moeller et al., 2006). Standard germination profile was followed at 0, 30, 60, 90 and 120 min of incubation by determining total colony formation (CFU_{total}), remaining heat resistant cells ("spores") after heat shocking at 80°C for 10 min ($CFU_{heat\ resistant\ cells}$), and optical density (OD at 600 nm). Colony formation ability (CFA) was determined from 100 µl aliquots of the suspension, plated on NB medium plates after appropriate dilutions in SAL (2.5) and incubated overnight at 37°C. Changes in the optical density were photometrically (spectrophotometer (U-3310, Hitachi, Tokyo, Japan) measured by analyzing 0.5 ml of each sample against the pure germination medium without microorganisms. For the study of the DNA repair capability of treated spores every 0.5 h a sample of 10 ml germination suspension was taken and centrifuged at 10 000×*g* for 5 min at 4°C; the supernatant was discarded and the pellet was stored at -80°C until further analysis e.g. DNA lesion characterization and gene expression profiling (i.e. RNA isolation).

2.1.7 DNA extraction

For characterizing UV-induced DNA photoproducts and oxidative damaged nucleobases, the DNA of irradiated and non-irradiated spores of the tested *Bacillus* sp. strains (2.2) was isolated and analyzed by sensitive and rapid HPLC-MS/MS technique (Douki et al., 2005a; 2005b; Pogoda de la Vega et al., 2005). This technique has allowed the separation, identification and quantitation of an entire suite of UV photoproducts, oxidative DNA lesions and further DNA damage at a level of resolution previously unattained.

DNA was isolated from the treated and non-treated, (dormant, germinated and outgrowing) *Bacillus* sp. spores using the Wizard Genomic DNA Purification Kit (Promega GmbH, Germany) procedure for genomic DNA isolation from bacteria with the following modifications. In order to remove the spore

coats, spores were centrifuged, resuspended in 10 ml of 50 mM Tris–HCl (pH 8.0), containing 8 M urea, 1 % sodium dodecyl sulfate, 10 mM EDTA, and 50 mM dithiothreitol, and incubated for 90 min at 60°C (Sargent, 1980). After washing thrice in SAL, the decoated spores (about 10^9/ml) were finally stored in SAL at 4°C. This method of chemical detaching of the spore coat layers is basic requirement for DNA extraction out of spores. Before adding lysozyme, the cells were suspended directly in lysis buffer and placed in a 2-ml tube with glass and ceramic beads from the FastDNA kit (Bio101, Vista, CA, USA). The tube was shaken for 45 s at the speed setting of 6.5 m/s in a bead beater (FP120 FastPrep cell disrupter; Savant Instruments, Inc., Holbrook, N.Y.) and incubation with RNAase and lysozyme was for 45 min at 37°C before the addition of the Protein Precipitation Solution (Promega GmbH, Germany). This procedure yielded about 1.0 - 3.0 µg DNA from each sample of treated and germinated spores (by using a spore concentration of 1×10^9). Due to the fact that a single *B. subtilis* 168 spore contains a single chromosome (Frenkiel-Krispin et al., 2004), which has a calculated weight of about 4.4 fg, the yield of spore DNA out of 1×10^9 spores should yield approximately 4.4 µg (in a hypothetical 100 % efficient isolation, W. L. Nicholson, pers. communication). The spore DNA extraction efficiency was of about 25 - 75 %. The isolated DNA from was air-dried after precipitation and stored at 4°C until further analysis.

2.1.8 RNA isolation and quantification

For RNA isolation of the germinating and outgrowing spores, samples (containing approx. 5×10^8 colony forming units) were centrifuged at 10 000*×g* for 5 min at 4°C, and the pellet was stored at -80°C until RNA isolation and analysis. RNA was isolated by the acid phenol method as described by a modified FastPrep protocol (QbioGene Company, MP Biomedicals, CA, USA). RNA extraction was performed at either 4°C or on ice. The frozen pellet was resuspended in 400 µl SAL containing RNAlater (QIAGEN, Duesseldorf, Germany), a RNA stabilization solution and transferred to a Lysing Matrix Tube B containing 0.1 mm silica spheres (QbioGene Company, MP Biomedicals, CA, USA) and 800 µl acid phenol solution (Carl Roth GmbH, Karlsruhe, Germany). The Lysing Matrix Tube was then shaken in the FastPrep Instrument (Eubio, Austria) for 45 s at a setting of 6.5 m/s, removed and centrifuged in a microcentrifuge (12 000*×g*, 5 min, 4°C). During all extracting and washing steps the upper phase, containing the RNA, was taken carefully to avoid transferring the debris- and DNA-containing pellets. The upper phase was transferred to an RNase-free 1.5-ml microcentrifuge tube

containing 800 µl acid phenol solution, shaken by hand for 60 s, and centrifuged (12 000*xg*, 5 min, 4°C). The upper phase was then transferred to a clean microcentrifuge tube containing 800 µl chloroform/isoamylalcohol (v:v, 96:4), shaken again and centrifuged as indicated above. Then the upper phase was transferred to a clean microcentrifuge tube and RNA precipitated by addition of 70 µl 3 M sodium acetate (pH 5.2) and 1 ml isopropanol (60 min, -80°C) followed by centrifugation (12 000*xg*, 5 min, 4°C). The pellet was washed with 1 ml ethanol, air-dried for 5 min, resuspended in 30 µl RNase-free H_2O (Invitrogen Corporation, Paisley, Scotland, U.K.). Isolated RNA was treated with RNase free DNase I to remove contaminating DNA. Purified total RNA was stored at -80°C. The purity of the RNA preparation was assayed spectrophotometrically from the 260 nm/280 nm ratio by two commercial methods: NanoDrop (NanoDrop Technologies, Willmington, DE, USA) using undiluted RNA preparation, and GeneQuant (Biochrom, Cambridge, U.K.) using a 1:10 dilution in RNase-free H_2O. The yield of the extracted RNA and intactness of rRNA was assayed from 1 µl of the diluted (1:10) RNA solution in a 2100 BioAnalyzer using the protocol of the RNA 6000 Nano Lab Chip Kit (Agilent, Böblingen, Germany). Only total RNA samples with a RIN (RNA integrity number) above 9.0 were used for further experiments (2.4.2).

2.2 Experimental conditions and exposure facilities

To examine genetic effects and the efficiency of DNA repair during spore germination, spores of *B. subtilis* were exposed to following DNA damaging agents: ionizing radiation (i.e. X-rays and selected accelerated heavy ions) and UV (i.e. mono- and polychromatic) radiation. For studying the effect of the environmental stress factors desiccation and shock pressure, spores were exposed to high vacuum ($>10^{-7}$ Pa) for inducing extreme dryness and for the astrobiological consideration of microbial survival after meteorite ejection processes (i.e. in order to determine the likelihood of bacterial endospores as potential candidates for "*Lithopanspermia*") spores were subjected to shock pressures in the range of 5 to 50 GPa.

2.2.1 Experimental strategy for studying DNA repair processes in *B. subtilis* spores

For studying the DNA repair of treated spores during germination a reticulating approach was used, i.e. all experiments were performed in a combination of various biochemical and molecular biological methods to characterize the interaction of following parameters:

- determination of the microbial survival,
- characterization of the DNA damage (induction, formation and repair),
- determination of mutational specifity,
- analysis of the transcriptional response.

Following sub-points describe the used standardized operation procedures for analyzing each selected treatment by detailing: the sample preparation and recovery, the used repair approaches, DNA damage characterization, assays for the determination of the resistance behavior, and the requirements of DNA microarray experiments.

2.2.2 Sample preparation

Spores of various *B. subtilis* strains (see Table 2-1, 2.1.2) were exposed to different types of radiation and pressure treatments. They were used either as spores in suspension or as dried spore (mono-)layers. Several critical features e.g. homogeneity was considered carefully for each sample preparation.

2.2.2.1 Air-dried spore (mono-)layers and sample recovery via "PVA-stripping"

For the determination of the spore resistance to various physical sporicidal agents, aqueous spores suspensions of different concentrations (Table 2-2) were spotted either on 7 mm diameter quartz discs (Heraeus Quarglas GmbH & Co.Kg, Hanau, Germany) or on 50×50 mm glass slides (neoLab, Migge Laborbedarf, Heidelberg, Germany), so that they spread and air-dried uniformly on the respective surface (as described in detail in Puskeppeleit et al., 1992). Immediately after sample preparation (spotting), all spore-containing glass surfaces (samples) were dried at 37°C under saturated humidity for 2 - 4 d for preventing droplet-formation and finally stored under laboratory conditions until further usages. With regard to homogeneity of the spore layers the quality of the air-drying was routinely checked by microscopic analysis.

To determine the sample ('target') distribution on the surface material and therewith their homogeneity following calculation was used: $Z = \frac{m \times e^{-m}}{1 - e^{-m}}$ *(Eq. 1)*

with $m = \frac{N \times f}{F}$; Z = fraction of free lying spores (%), N = spore concentration, f = area of the biological target (a single spore has a spherical shape with a diameter of about 1 µm, resulting in a spore area of 0.79 μm^2) and F = sample surface area of the to be spotted material. Table 2-2 gives the fraction of free lying spores for different spore concentrations.

Table 2-2 Fraction of single free lying spores, calculated in percent (%).

Spore concentration / surface material	1×10^7 spores	1×10^8 spores	1×10^9 spores	5×10^9 spores
(i) 7 mm diameter-quartz disc (QD)	90.09	30.23	0.00	0.00
(ii) 50×50 mm glass slide (GS)	99.84	98.43	85.03	40.99

To recover the spores, the dry (mono-)layers were covered by a 10 % aqueous polyvinyl alcohol solution (PVA; obtained from DLR, Cologne, Germany) and air-dried under laboratory conditions for 1 - 2 d. After drying the spores-PVA layer was stripped off as described previously (Horneck and Bücker, 1983) and subsequently resuspended in 1 ml sterile distilled water, resulting in > 95 % recovery of the spores (Horneck and Bücker, 1983; Horneck et al., 2001a; 2001b). This procedure (later called 'PVA-stripping') has no geno- or cytotoxic effects on the vitality of the spores (Horneck et al., 2001b). For each treatment at least three replica samples (i.e. quartz disc or glass slide) were analyzed.

2.2.2.2 Spores in aqueous suspension

Spores in suspensions were exposed to UV radiation and X-rays. During the irradiation each spore suspension was stirred continuously to ensure homogeneous exposure. Furthermore, the non-coverage of the spores was calculated for each spore concentration using (*Eq. 1*) and can be seen in Table 2-3.

As container for all radiation experiments sterile Petri dishes (Nunc A/S, Roskilde, Denmark) were used.

Table 2-3 Fraction of uncovered spores in suspension, calculated in percent (%).

Spore concentration / Petri dish size	1×10^7 spores	1×10^8 spores	1×10^9 spores	5×10^9 spores
(iii) 10 cm diameter	99.95	99.50	95.06	76.95

2.2.3 UV irradiation

Spores were irradiated either with mono- or polychromatic UV radiation. Monochromatic UV irradiation was performed via a low-pressure mercury lamp and polychromatic UV radiation was applied by using a Xenon short arc lamp (artificial sunlight simulator) and optical filter combinations.

All irradiations with artificial UV, low-pressure mercury lamp and artificial sunlight simulator, took place in a black-painted UV laboratory at the German Aerospace Center, Institute of Aerospace Medicine, Radiation Biology Division (DLR, Cologne, Germany). To ensure optimal conditions for each irradiation all manufacturer information e.g. burn-in of the UV lamps for 1 h, were followed. UV dosimetry was performed using a calibrated UVX-radiometer (UVP Ultra-Violet Products, Cambridge, UK) and a spectroradiometer (i.e. Bentham model DM 300, Reading, UK).

2.2.3.1 UV-C irradiation of *B. subtilis* spores

Spores ((i) 10 ml aqueous suspension 10^8 spores/ml or (ii) air-dried spore monolayer on either 7 mm quartz discs (10^7 spores/QD) or 50×50 mm glass slides (10^9 spores/GS)) were exposed to UV-C radiation from a low-pressure mercury lamp (Model NN 8/15, Heraeus, Berlin, Germany). All irradiations were carried out at 4°C while the suspensions were continuously stirred to ensure homogeneous exposures. The spectral irradiance of the mercury low-pressure lamp (NN 8/15, Heraeus, Berlin, Germany) with a major emission line at 253.65 nm (Fig. 2-1) was measured with a double monochromator (Bentham model DM 300). A fluence rate of 90 µW/cm² was determined at the sample site by using a UV-X radiometer with a UV-sensor (UVX-25) for 254 nm (both UVP Ultra-Violet Products, Cambridge, UK). After UV-C irradiation at defined fluences following aliquots were taken

from the suspension for further analysis: 100 µl for survival studies, 1.5 ml for DNA photoproduct analysis (DNA extraction procedure is described in 2.1.7), and separately 10 ml (exposed to a fluence of 125 J/m^2) for germination and repair studies (2.1.6, 2.1.7, 2.3.1 and chapter 2.4), spores in monolayers were resuspended as described in 2.2.2.1 and treated accordingly. Survival was determined from appropriate dilutions in distilled water from colony forming units (CFU) after overnight growth on nutrient broth (NB) agar plates at 37°C (2.1.5).

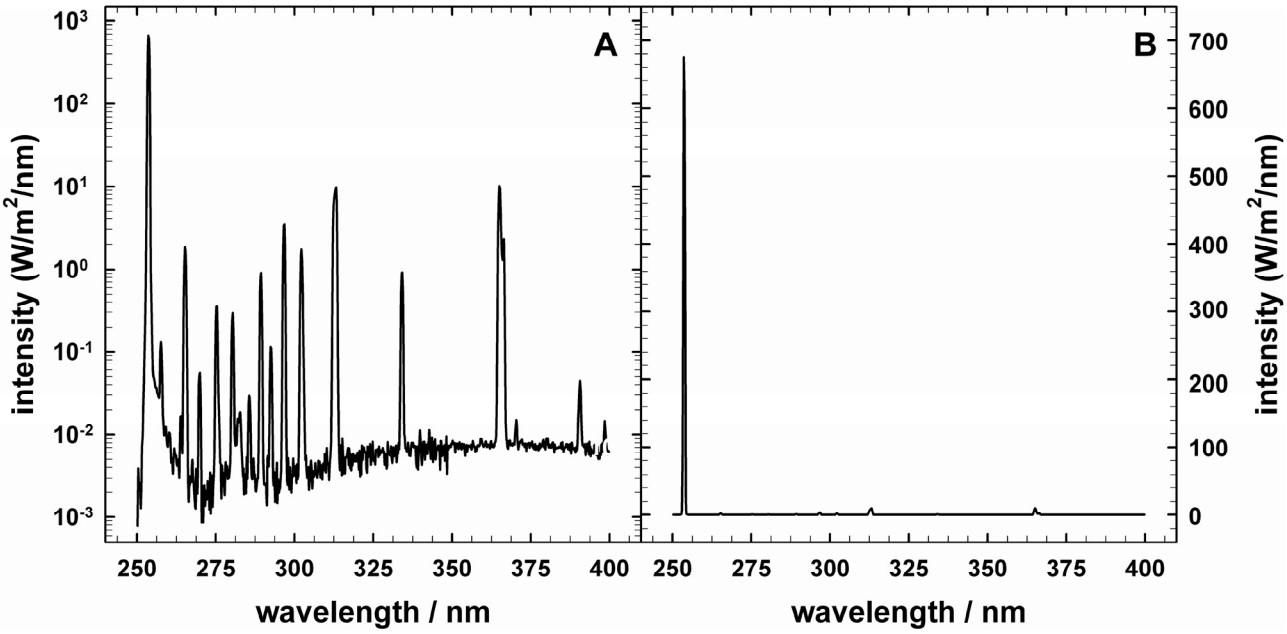


Fig. 2-1 Spectral irradiance, semi-logarithmic (**A**) and linear (**B**) plotting, of the used mercury low-pressure lamp (NN 8/15, Heraeus, Berlin, Germany). The major emission line is at 253.65 nm. The spectral data were kindly provided by Dr. R. Facius (DLR, Cologne, Germany).

2.2.3.2 Polychromatic UV irradiation of *B. subtilis* spores

Spores ((i) 10 ml aqueous suspension 10^8 spores/ml or (ii) as air-dried spore monolayer on either 7 mm quartz discs (10^7 spores/QD) or on 50×50 mm glass slides (10^9 spores/GS)) were exposed to defined spectral ranges of UV-(A+B) or UV-A radiation obtained by a 1000 W Xenon short arc lamp (Polytech, Waldbronn, Karlsruhe, Germany) and optical filter combinations (Schott AG, Mainz, Germany). All irradiations were carried out either at 4°C while the suspensions were continuously stirred to ensure homogeneous exposures or as irradiation of spore monolayers on appropriate sample carriers. The spectral irradiance (Fig. 2-2) of the Xenon short arc lamp was measured with a double

monochromator (Bentham model DM 300). After UV irradiation at defined fluences aliquots were taken for further analysis as described in 2.2.3.1. Survival was determined from appropriate dilutions in distilled water from colony forming units (CFU) after overnight growth on nutrient broth (NB) agar plates at 37°C (2.1.5).

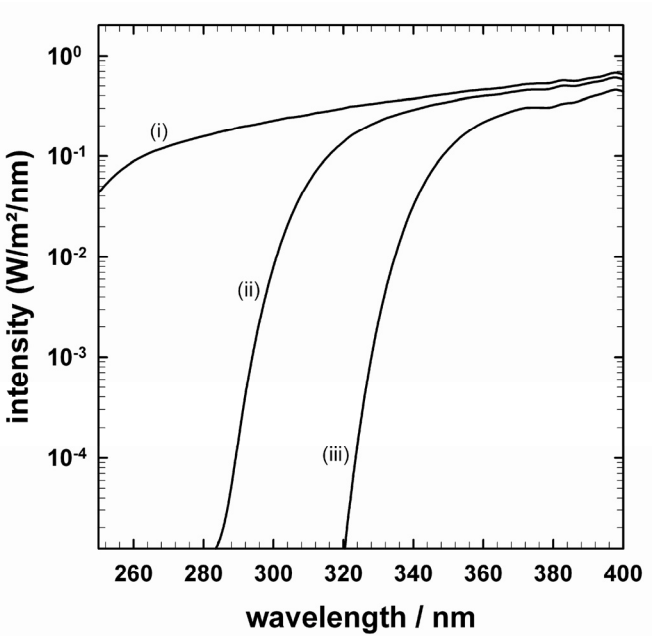


Fig. 2-2 Spectral irradiance, semi-logarithmic plotting, of the used 1000 W Xenon short arc lamp ((i); Polytech, Waldbronn, Karlsruhe, Germany) and the used optical filter combinations (WG filters for (ii) UV(A+B, > 290 nm) and (iii) UV(A, > 320 nm); Schott AG, Mainz, Germany). The spectral data were kindly provided by Dipl.-Ing. J. Drescher (DLR, Cologne, Germany).

2.2.4 Exposure to ionizing radiation

Purified spores of several *B. subtilis* strains (2.1.2) were irradiated with X-rays and accelerated heavy ions (of different linear energy transfer LET). Spores were spotted on two different units: (i) on 7 mm diameter quartz discs ($(1\times10^8$ spores/40 µl) for colony formation; Heraeus Quarglas GmbH&Co.Kg, Hanau, Germany; as described in detail in Puskeppeleit et al., 1992) and (ii) on 50×50 mm glass slides ($(5\times10^9$ spores/500 µl) for gene expression studies and mutation induction), resulting in a equable spreading on the respective surface. The spore loaded materials were placed in sterile Petri dishes (Nunc A/S, Roskilde, Denmark), closed and these packages ('exposure units') were exposed to ionizing radiation. The spores were irradiated as air-dried monolayers with X-rays (2.2.4.1) and accelerated heavy ions (2.2.4.2). Spore recovery from the irradiated (air-dried) spores was performed as described above (2.2.2.1). After exposure to ionizing radiation (2.2.4.1 and 2.2.4.2), spore survival via a colony formation assay (2.1.5), characterization of induced DNA damage (2.3.2), changes in their antibiotic

resistance to rifampicin (2.3.3) and transcriptional profile during spore germination (2.3.4 and chapter 2.4) were determined and characterized in detail.

2.2.4.1 X-rays

For studying the impact of X-rays induced damage on the spore survival, air-dried spore layers ('exposure units' of (i) and (ii)) were irradiated at room temperature with X-rays (150 keV/19 mA). X-rays irradiation was generated by an X-ray tube (Mueller Type MG 150, MCN 165, Philipps, Hamburg, Germany). Radiation dosimetry and dose calculations were used as described in detail by Micke et al. (1994). The determination of the spore survival (2.1.5), recording of the induced changes in the DNA (2.3.3) and further genetic analyses were performed as described in 2.3.4 and chapter 2.4.

2.2.4.2 Heavy ions irradiation

In the framework of this thesis, irradiations with four heavy ions (Table 2-4) were performed at the **H**eavy **I**on **M**edical **A**ccelerator (HIMAC) at the **N**ational **I**nstitute for **R**adiological **S**ciences (NIRS) in Chiba, Japan. A large and uniform irradiation field is provided for biological and biomedical research. The data was gathered during three exposure periods starting in summer 2005 and lasting until autumn 2006. Efficiency functions in dependency on ion species and linear energy transfer (LET) in the LET range from 2 - 200 keV/µm (Table 2-4) were recorded for studying the inactivation, mutagenesis and DNA repair capability. Detailed information concerning the irradiation geometry of the HIMAC facility, beam monitoring, dosimetry and dose calculations were described in detail in Okayasu et al. (2006) and in the PhD thesis of Thomas Berger (Berger, 2003; doctoral thesis, Technical University of Vienna, Austria). The diameter of the beam is 100 mm. Maximum intensity is between 3 - 5 Gy/min depending on Bragg Peak (the maximum dose of an accelerated particle, before the particle loses all its energy by dose deposition along its ionization path and stops).

Access for investigators in the field of radiobiology and radiation protection to the HIMAC is limited and only possible by means of an accepted research proposal. The proposal "Gene activation of heavy ion treated *Bacillus subtilis* 168 endospores during germination involved DNA-repair (17B463)" was approved and a project for the irradiation with heavy ions was started in summer 2005 (http://www.nirs.go.jp/news/event/2006/04_03/biology.shtml). Irradiations at HIMAC were performed

in the Biology Room of the facility. For all of the experiments at least three exposure units of both types ((i) and (ii)) for each tested strain were used to reduce statistical uncertainties. As non-irradiated (mock-treated) controls a laboratory control (at DLR, Cologne, Germany) and a "flight control" (DLR-HIMAC-DLR) were used. There were no significant differences in the colony formation of the laboratory control and the "flight control" observable after analyzing of the heavy ion irradiated samples. The exposure units were placed on polyethylene matrices of the dimension 35×25×1 cm³ in the Biology Room at HIMAC. Irradiations were performed directly in the beam line, using no absorber material to ensure equal energies of the chosen ions. Recovery of the spores and further sample analysis was performed as described in 2.2.2.1 and 2.2.4.

Table 2-4 Ions and energies for the spore inactivation

Ion	Energy (MeV/n)	LET (keV/µm)	Intensity (particles/s)
Helium (He)	150	2.2	1.2×10^{10}
Carbon (C)	400	12	2.0×10^{9}
Silicon (Si)	490	50	4.4×10^{8}
Iron (Fe)	500	200	2.5×10^{8}

For comparison, spores were irradiated with X-rays. X-rays were obtained at the German Aerospace Center (DLR), Cologne, Germany (Baltschukat et al., 1986; Baltschukat and Horneck, 1991). Standard irradiation procedures (2.2.4.1) concerning beam monitoring, dosimetry and dose calculations were performed as described in detail elsewhere (Baltschukat et al., 1986; Micke et al., 1994).

2.2.5 High vacuum exposure

Spores of various *B. subtilis* species (2.1.2) were exposed to extreme low pressure for studying the spore resistance to extreme dryness induced by high vacuum (Munakata et al., 1997). Spores were spotted on two different units: (i) on 7 mm diameter quartz discs ((1×10^{8} spores/40 µl) for determination of survival) and (ii) on 50×50 mm glass slides ((5×10^{9} spores/500 µl) for gene expression studies and mutation induction), so that they have an uniform distribution on the respective surface after drying. Air-dried spore-monolayers were exposed to high vacuum produced by a ion getter pumping system (400 l/s; Varian s.p.a. Via Varian, Leini, Torino, Italy) reaching a final pressure

of 10^{-7} Pa (according to Horneck, 1993; Munakata et al., 1997). After vacuum exposure at defined times (15, 30 and 450 d), the following aliquots were taken for further analysis: a survivability assay (2.1.5), mutagenesis assay (2.3.3) and transcriptional profiling after 1 h germination (2.3.4 and chapter 2.4). Spore recovery from the vacuum exposed (extreme dried) spores was performed as described above (2.2.2.1).

2.2.6 Shock pressure experiments

In the framework of this thesis, shock pressures experiments were performed at the Fraunhofer Institute for High-Speed Dynamics, Efringen-Kirchen, Germany (EMI, Ernst-Mach-Institute) to experimentally test the likelihood of interplanetary transfer of (microbial) life inside meteorites between Mars and Earth (or Mars-like planet to other life-sustaining exoplanets). Bacterial endospores (of *B. subtilis*; investigated in this thesis), endolithic cyanobacteria (*Choococcidiopsis* sp. 029; analyzed by Prof. Dr. C. S. Cockell, Open University, Milton Keynes, UK) and epilithic lichen (*Xanthoria elegans*; investigated by Dr. J.-P. P. de Vera, University of Düsseldorf, Germany) embedded in various Martian host rock and soil analogous material were exposed to shock pressures between 5 and 50 GPa which is the range of the pressure observed in Martian meteorites (Fritz et al., 2005) and the survivability of shock pressure treated biological systems (in this thesis spores of *B. subtilis* were used) was studied. The data was gathered during 2003 - 2006 in the frame of two German Research Foundation (DFG) research projects “Meteorite ejection and microbial life (1) and (2)” (DFG HO 1508/3-1, DFG STO 101/39-1/2 and DFG RA 1049/1-2). Both DFG project were approved and funded by the DFG Priority Program 1115 “Mars and the terrestrial planets” (SPP 1115; Main coordinator is Prof. Dr. T. Spohn).

In the shock recovery experiments using a high-explosive plane wave set-up (Stöffler and Langenhorst, 1994; Horneck et al., 2001b; Fig. 2-3) a suite of defined shock pressures of 5, 10, 20, 30, 41.5, and 50 GPa was applied on different (rock and soil) material with embedded layers of viable microorganisms (sample preparation was modified after 2.2.2.1).

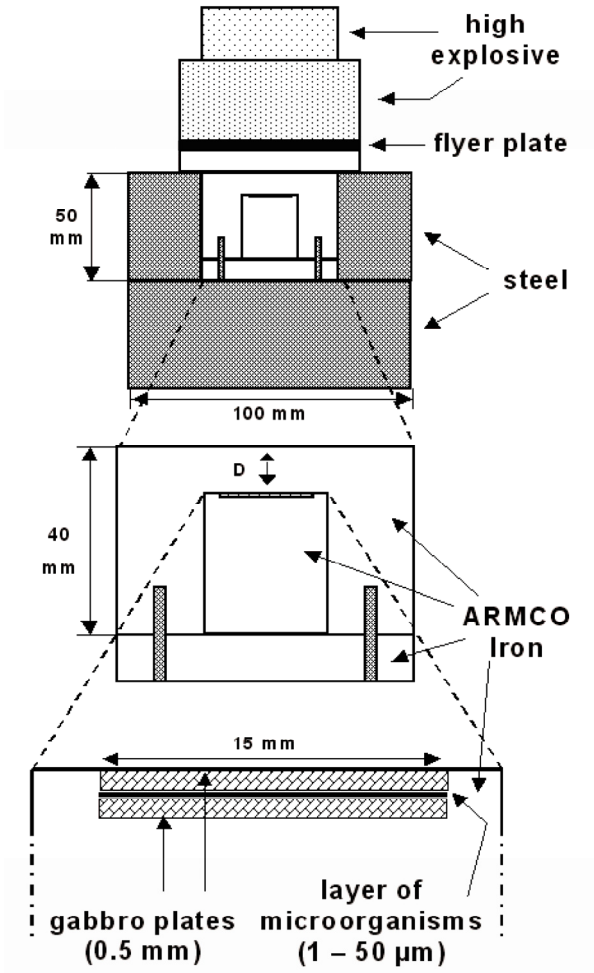


Fig. 2-3 Experimental set-up for the shock recovery experiments performed at the Ernst-Mach-Institute for High-Speed Dynamics, Freiburg, Germany. Thin dry layers of microorganisms were embedded between two plates of rock material (in this scheme gabbro) and subjected to shock pressures between 5 and 50 GPa using a shock reverberation technique with an accuracy of ± 3 % (Stöffler and Langenhorst, 1994). This sketch was kindly provided by Dipl. min. chem. C. Meyer.

The nominal shock pressures were calculated (by Dipl. min. chem. C. Meyer, Museum for Natural History, Mineralogy Division, Humboldt-University of Berlin, Germany) on the basis of the shock reverberation technique (Stöffler and Langenhorst, 1994) and checked by refractive index measurements of particle grains from the shocked and recovered rock material (Stöffler et al., 1986). In the reverberation technique used in these experiments, the maximum shock pressure is achieved by a sequence of increasingly stronger shock waves reflected at the steel-rock interfaces whereas the temperature is induced predominantly by the first "low pressure" shock wave in the rock material. The

calculated shock temperatures prevailing in the rock sample for less than 1.4 µs were relatively low in these experiments (ΔT = 1 to 100°C for 5 to 50 GPa; Table 2-5).

The post-shock temperature increase (ΔT = 1 to 60°C; Tab. 2-5) lasted for a few minutes and probably increased to a final temperature (ΔT = 1 to 304°C) which is determined by heat exchange with the steel container. Moreover, the few µm thick microbial layer experienced maximum temperature spikes (e.g., ΔT = 500 and 1000°C at 18 and 43 GPa, respectively, as calculated for a 50 µm thick layer of water by Dr. B. A. Ivanov, pers. comm.) for less than 1 µs due to its low shock impedance. In reality, this temperature is probably much lower because the spore layer is about 2 µm thick and spores have an extremely low water content (Nicholson et al., 2000).

Table 2-5 Pressure and temperature conditions in shock experiments with spores embedded in gabbro

Shock pressure (GPa) in gabbro, based on the refractive index	Final shock temperature in gabbro (°C)	Final post-shock temperature in gabbro (°C)[a]
5	<1	<1
10	1	7
15	3	22
20 ± 4	7	39
32 ± 3	23	109
42 ± 3	53	210
50 ± 3	99	304

[a]after heat exchange with ARMCO iron container (calculation by Dr. B. A. Ivanov, Institute for Dynamics of Geospheres, Russian Academy of Science, Moscow, Russia).

2.2.6.1 Sample preparation and recovery of the shocked host rock and soil material

Spores (1×10^8/100 µl) of *B. subtilis* TKJ6312 (for "Meteorite ejection and microbial life (1)") and of *B. subtilis* RM01 (for "Meteorite ejection and microbial life (2)") were spotted on 15 diameter mm rock discs, following rock material was used: gabbro, dunite and sandstone (mineralogical composition can be see Fig. 2-4) and dried as described previously (2.2.2.1), so that the spores were spread uniformly on the respective rock material. To simulate the Martian evaporite component 1 ml spores (1×10^9) were diluted in 9 ml of a 30 % NaCl solution (Merck KGaA, Darmstadt, Germany), transferred to a sterile

mortar and air-dried until the water was evaporated. After drying the spores-halite mixture was grinded until a homogenous powder was produced. The spore concentration of this powder (later called halite-spores-powder; Fig. 2-4) was determined by using a colony formation assay (2.1.5) and stored in closed reaction tube under laboratory conditions. Spores (1×10^{10}/ml) were also mixed with a simulated Martian regolith (obtained from Prof. D. Stöffler, Museum for Natural History, Mineralogy Division, Humboldt-University of Berlin, Germany and later called regolith-spores-powder), dried, grinded and processed as described before for the halite-spores-powder (Fig. 2-4), resulting in a spores concentration of 1×10^{9} per soil powder material.

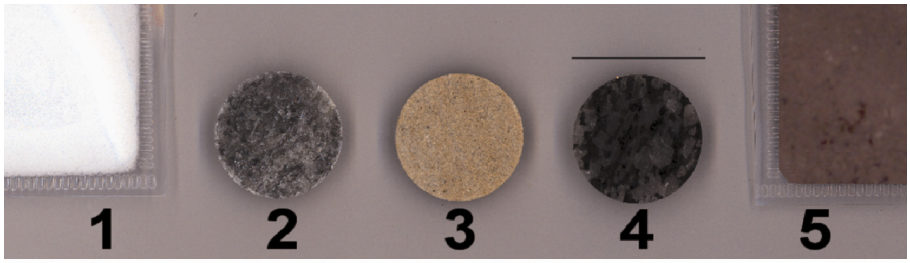


Fig. 2-4 Martian host rock (meteorite) material (**2** dunite [corresponding to the Martian Chassignite meteorites (intrusive, 84 % olivine, 11 % othropyroxene and minor phases of chromite, magnetite, ilmenite and pyrrhotite); rock sample of Aheim, Norway], **3** sandstone [metamorphic sandstone (71 % quartz and trace of plagioclase; porosity of 11 % and pore size of 0.05 to 1.00 mm, complete recrystallization); sample origin was Hesse, Germany] and **4** gabbro [analogue for the relatively coarse-grained basaltic Martian Shergottites meteorite (69 % plagioclase, 27 % ortho- and clinopyroxene, and traces of quartz); obtained from the Bushveld compley (eucritic basalts), South Africa]) and soil analogue (**1** halite [99.5 % NaCl; Merck KGaA, Darmstadt, Germany] and **5** MRS07 [simulated Martian regolith MRS07 (47.7 % Na-montmorillonite, 9.9 % kaolinite, 21.3 % hematite (19.2 % Fe_2O_3 with 1.3 % SiO_2), 13.0 % anhydrite, 7.1 % $MgSO_4$, 1.0 % NaCl, 2.5 % Na_2O, 3.4 % MgO, 14.1 % Al_2O_3, 34.6 % SiO_2, 5.1 % SO_3, 0.2 % Cl, 0.2 % K_2O, 6.1 % CaO, 0.1 % TiO, 18.5 % FeO; kindly provided by Prof. D. Stöffler, Museum for Natural History, Mineralogy Division, Humboldt-University of Berlin, Germany]) used in the shock pressures experiments. Scale bar (above **4**) is 15 mm.

In case of the rock material, spores were spotted on the lower rock disc, covered with the upper unspotted rock disc, resulting in a rock discs-spores sandwich and placed in the indentation of the high-grade stainless steel (ARMCO iron) shock cylinder. Both spores-powder (i.e. halite and regolith) were filled in the indentation of the shock cylinder, compressed and filled until a planar surface area was created and assembled in the high-explosive plane wave set-up (Fig. 2-3).

All shock pressures experiments were performed at the Fraunhofer Institute for High-Speed Dynamics, Efringen-Kirchen, Germany (in cooperation with Dr. U. Hornemann). As seen in Fig. 2-3 the flyer metal plate was accelerated (either by high explosives or by a light-gas gun) to velocities up to 2.6 km/s and impacted on the ARMCO iron container (Stöffler and Langenhorst, 1994; Horneck et al., 2001b; 2007). The resulting shock wave which arrives at the upper iron, rock interface with a defined pressure propagates into the shocked material and decreases its pressure to a lower pressure because of the lower shock impedance (density of the shocked material at zero pressure times shock velocity) of the shocked material. The shock is reflected at the lower interface of the shocked material and the ARMCO iron and runs back into the rock material, then gets reflected back again into the shocked material and the ARMCO iron. These reverberations and stepwise increases of the pressure continue until the rarefaction wave reflected back from the back of the impacted flyer plate arrives at the shocked material sample, thereby releasing the sample from the compressed state.

Shock pressures in the range of 5 - 50 GPa were applied by using high explosive approaches. To vary the final shock temperature of some shock pressure experiments were conducted at three different initial starting temperatures (193, 233 and 293 K) by using a cryo-device or frozen carbon dioxide (dry ice). After the shock pressure experiment was carried out and mechanically opened at the workshop of the German Aerospace Center, Institute of Aerospace Medicine, Cologne, Germany it was stored at 4°C until further analysis.

For recovering spores from the shocked material two approaches were used: in the case of the rock discs-spores sandwich, the upper disc was removed and both discs (upper and lower) were cover with a PVA solution, air-dried and stripped off as described previously (2.2.2.1). The ‘PVA-stripping’ was repeated at least five times repeated, the PVA-spores-layers were collected, resuspended in sterile water and stored at 4°C for further analysis. Powdery shocked material (e.g. halite) was collected, weighed for mass determination, resuspended in sterile water for further analysis and processed as described previously. The spore survival was determined as colony forming ability on NB medium plates (as described in 2.1.5). Contamination of the spores-samples was excluded by using a genetic marker test (Horneck et al. 2001a; 2001b; 2007) i.e. for spores of *B. subtilis* TKJ 6312 (with a histidine, methionine and leucine auxotrophy) and for spores of *B. subtilis* RM01 (carrying a codon change in the

rpoB gene, leading to a defined increased rifampicin resistance). The deficiency markers allowed discriminating the test samples against possible contaminations by a marker test e.g. growth on minimal medium plates (SMM plates, with/without auxotrophy-essential supplements, as described in detail by Horneck et al. (2001b)) or parallel growth on high rifampicin concentrated NB medium plates (50 µg/ml) in adaptation of the standard colony formation assay (2.1.5 and 2.3.3). Furthermore, aliquots of each shocked material was given to the Museum for Natural Histrory, Mineralogy Division, Humboldt-University of Berlin, Germany for mineralogical studies e.g. refractive index measurements of the shocked material (e.g. plagioclase grains) to determine the nominal shock pressure (Meyer, 2005; diploma thesis, Humboldt-University of Berlin, Germany).

2.3 Characterization of induced DNA damage

The standard analysis protocol for each treated spore sample included tests of (i) survival, (ii) mutation induction, (iii) DNA damage analysis, and (iv) transcriptional responses during germination. After irradiation of spores with UV (UV-C, UV-A) or ionizing radiation (X rays, accelerated heavy ions) (2.2.3.1, 2.2.3.2 and 2.2.4.2) the DNA was isolated from the spores and the induced DNA damage (particularly DNA bipyrimidine photoproducts induced by UV or oxidative base damage induced by ionizing radiation) was analyzed by HPLC-MS/MS. To determine the efficiency and kinetics of repair of DNA base damage during germination of irradiated spores, DNA was also extracted at 30 min intervals of germination for HPLC-MS/MS analysis.

2.3.1 Quantitative analysis of inter- (and intrastrand) bipyrimidine photoproducts

Directly after UV irradiation as well as subsequently during germination at selected repair time points samples (of dormant, germinating or outgrowing spores) were taken for DNA photoproduct analyses, the DNA was isolated (2.1.6 and 2.1.7) and air-dried. Dry pellets of isolated DNA were suspended in 50 µl of deionized water. After resuspending, the DNA was hydrolyzed by incubation for 2 h at 37°C at pH 5.5 in the presence of nuclease P1 (0.5 U) and phosphodiesterase II (0.01 U). The pH was adjusted to 8 (with 1 M NaOH) and alkaline phosphatase (2 U) and phosphodiesterase I (0.05 U) were added. After 2 h incubation at 37°C 3.5 µl 0.1 N hydrochloric acid (HCl) were added to the DNA nucleotide solution. The samples were subsequently centrifuged (5 000 *x g*, 5 min, room temperature) and the aqueous phase was collected prior to be transferred into HPLC injection vials. The samples were

freeze-dried and resolved in 30 µl 0.02 M triethylammonium acetate. The amount of the main bipyrimidine photoproducts, measured as modified dinucleoside monophosphates (an assortment of standardized UV-detectable DNA photoproducts is shown in Fig. 2-7), was determined by HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) (Ravanat et al., 2001; Douki et al., 2005a; 2005b; Pogoda de la Vega et al., 2005). A detailed overview on analytical working flow of UV-damage DNA is shown in Fig. 2-5.

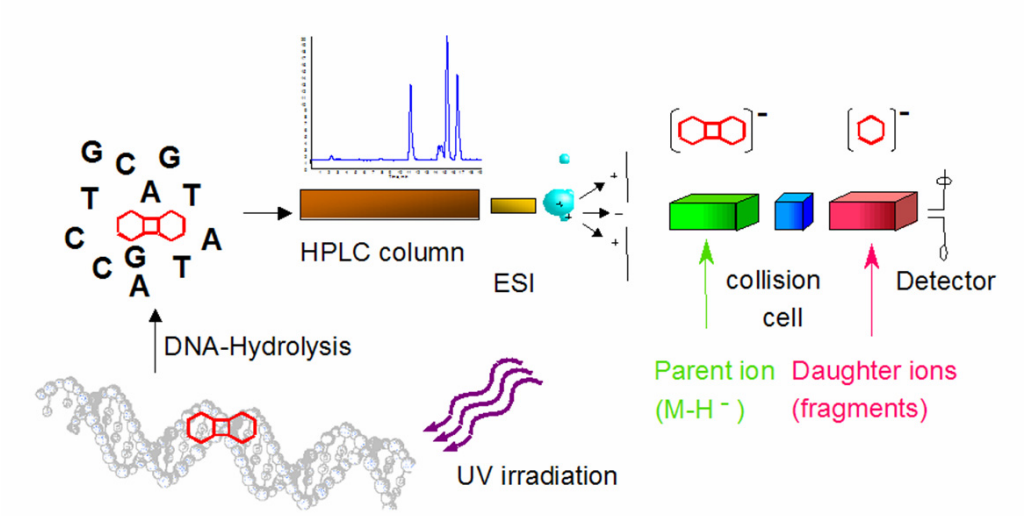


Fig. 2-5 Configuration of the HPLC coupled to tandem mass spectrometry (HPLC-MS/MS) and the analytic process to determine UV-induced DNA photoproducts. The scheme was kindly provided by Dr. T. Douki.

The samples were injected on an Uptisphere (3 µm particle size, 150×2 mm) octadecylsilyl silica gel column (Interchim, Montluçon, France) connected to a series 1100 Agilent chromatographic system. The mobile phase was a gradient of acetonitrile in 2 mM aqueous triethylammonium acetate. At the outlet of the column, the eluent was mixed with methanol (flow-rate 100 µl//min) and directed on-line towards the inlet of an electrospray API 3000 triple quadrupole mass spectrometer (SCIEX/Applied Biosystems, Toronto, Canada). Specific transitions corresponding to TT, TC, CT and CC cyclobutane pyrimidine dimers (CPDs), (6-4) photoproducts (6-4PPs) and Dewar valence isomers as well as the spore photoproduct SP (THDT) were monitored (chemical structures of CPD, 6-4PP and SP are shown in Fig. 2-6).

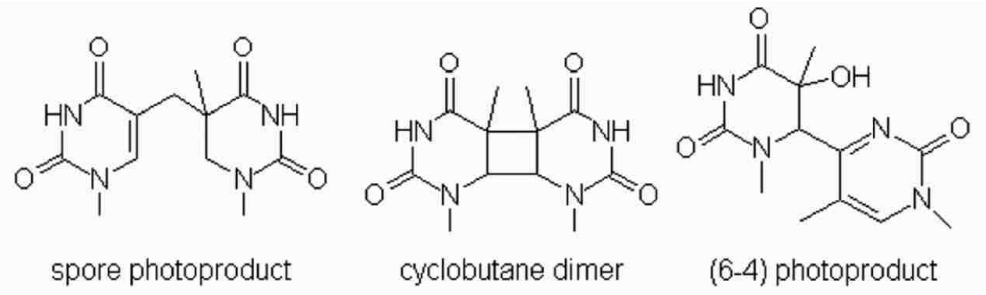


Fig. 2-6 Structure of 5-thyminyl-5,6-dihydrothymine spore photoproduct SP, cyclobutane pyrimidine dimer CPD, and pyrimidine (6-4) pyrimidone photoproducts 6-4PP formed between adjacent thymine residues in DNA. In contrast to UV irradiation of vegetative cells that generates cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) between adjacent pyrimidines as predominant DNA lesions, irradiation of spores produces little CPD but generates almost exclusively SP (Nicholson et al., 2000; Douki et al., 2005a; 2005b).

The response of the detector was calibrated by injection of known amounts of authentic photoproducts (see Fig. 2-7). Amount of analyzed DNA was inferred from the intensity of the peak corresponding to normal nucleosides on a chromatogram recorded by a UV spectrophotometer placed on-line before the inlet of the mass spectrometer as described by Douki et al. (2005a; 2005b).

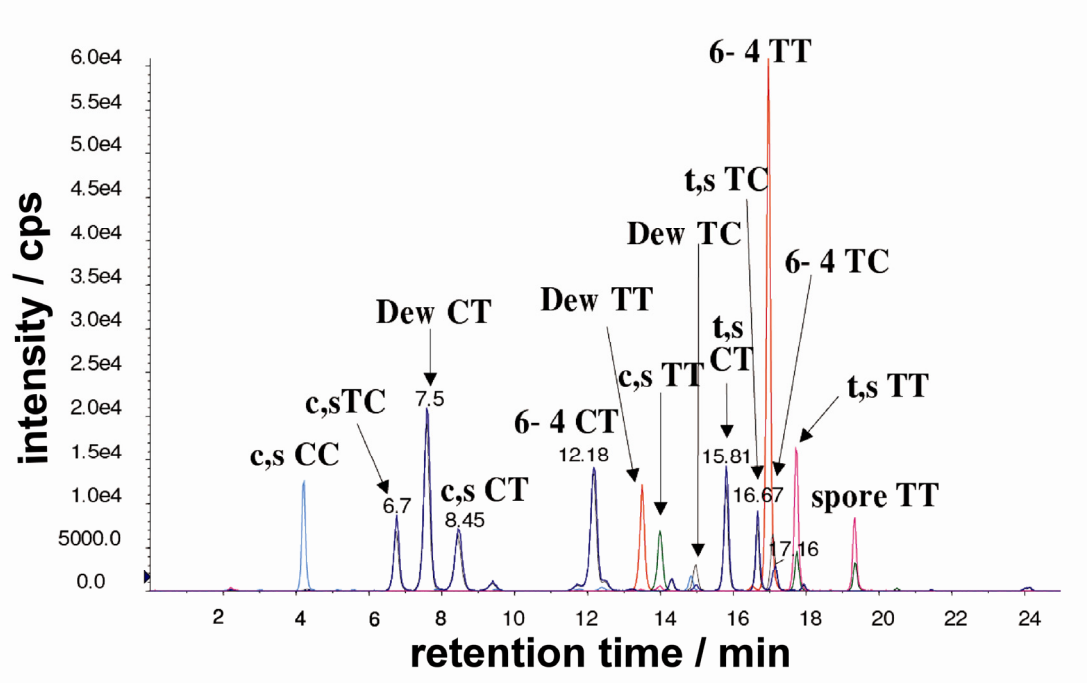


Fig. 2-7 Mass spectrometry chromatogram of all detectable DNA bipyrimidine photoproducts. The chromatogram was kindly provided by Dr. T. Douki.

2.3.2 Determination of nucleobase modifications as oxidative DNA damage

DNA extracted from irradiated (X-rays or heavy ions) spores was hydrolyzed into nucleosides according to a reported method (Frelon et al., 2000). DNA solutions (50 ml) were adjusted at pH 5.5 by addition of 5 ml of buffer (100 mM succinic acid, 50 mM $CaCl_2$, 5 μM $ZnSO_4$, pH 5.5). Samples were then incubated for 2 h at 37°C in the presence of 2.5 U of nuclease P1 and 0.05 U of phosphodiesterase II. Then, Tris buffer (6 ml, 500mM Tris, 1 mM EDTA, pH 8) was added together with 2 U of alkaline phosphatase and 0.05 U of phosphodiesterase I. The sample was then incubated for 2 h at 37°C. Hydrochloric acid (3 ml, 0.1 N) was added and the tubes were centrifuged at 7 000×*g* for 5 min. The digested samples (injection volume 40 ml) were analyzed by HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) for their content in oxidized nucleosides. HPLC separations were performed on a 2×150 mm octadecylsilyl silica gel (3 mm particle size) column (Uptisphere, Interchim Montluçon, France) connected to an Agilent Series 1100 HPLC system (Palo Alto, CA, USA). A gradient of acetonitrile in a 2 mM aqueous solution of ammonium formate was used. Ions of the DNA lesions of interest were produced in the electrospray ionization probe of an API 3000 triple quadrupole mass spectrometer (SCIEX/Perkin-Elmer, Thornhill, Canada). Thymidine glycols (ThdGly) and 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodGuo) (Fig. 2-8) were quantified either in the positive or negative ionization mode using MS/MS transitions reported elsewhere (Frelon et al., 2000). Isotopically labelled internal standards were used for isotopic dilution quantification of the lesions in cellular DNA. The retentions times of the oxidized nucleosides were the following (in min): ThdGly 3.4 and 8-oxodGuo 27.3. The amount of DNA analyzed was calculated from the area of the 20-deoxyguanosine peak (19.8 min) displayed in the HPLC elution profile. This was achieved using an ultraviolet (UV) spectrometer (λ = 285 nm) placed at the outlet of the column. In a few experiments involving cellular DNA, 8-oxodGuo was also quantified by HPLC associated with electrochemical detection (HPLC-EC) as previously reported (Douki et al., 1997; 2006).

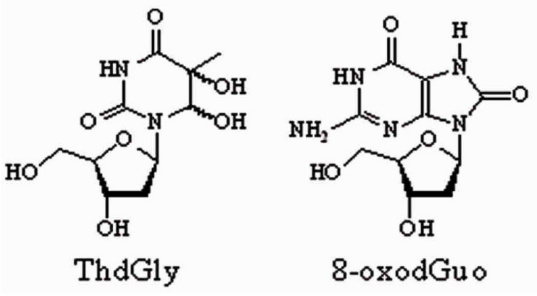


Fig. 2-8 Chemical structure of the studied oxidized nucleosides: thymindine glycols (ThdGly) and 8-oxo-7,8-dihydro-20-deoxyguanosine (8-oxodGuo) (Douki et al., 2006).

2.3.3 Assays for mutation induction to rifampicin resistance

For studying the mutagenic effects of ionizing radiation (2.2.4) and high vacuum (2.2.5) exposure to rifampicin-resistant (Rif^r) treated (and non-treated) spores were plated in appropriate dilutions in sterile distilled water on NB medium plates (2.1.5) containing 50 µg/ml rifampicin (Merck KGaA, Darmstadt, Germany). Rif^r-mutants arose after overnight incubation at 37°C and they were then counted. The frequency of the rifampicin mutations was expressed as the number of resistant colonies divided by the number of viable spores plated. Rif^r mutants were selected as an indication of induced DNA damage. The mutagenic specificity was analyzed by PCR as follows: Rif^r-colonies were picked and incubated in 5 ml NB medium (containing 50 µg rifampicin/ml) overnight at 37°C, 1 ml culture were centrifuged at 12 000 *×g* for 5 min at 4°C, and the pellet was resuspended with 0.2 ml TE buffer (10 mM Tris-HCl, 1 mM Na_2EDTA; pH 8.0). Template chromosomal DNA was prepared by heating the cell suspension at 95°C for 10 min, cooled down to room temperature, and 0.2 ml chloroform/isoamylalcohol (v:v, 96:4; Merck KGaA, Darmstadt, Germany) were added, shaken by hand for 1 min, and centrifuged at 14 500 *×g* for 90 s in a microcentrifuge for removing cellular debris. Then the upper phase was transferred to a clean microcentrifuge tube and stored at 4°C until further analysis. The region of *rpoB* was amplified by PCR using following primer pair (5'-ACGGAGTAGGCGACACAGAT and 5'-AACGAGCTACGATGCTGTCA) flanking a 609-bp fragment (fragment position in *rpoB*: 1178 - 1766 bp) containing the rifampicin resistant cluster I. Each PCR reaction was performed on 5 µl template DNA (0.2-0.15 µg DNA/µl), with each 1 µl pmol of the primer pairs, 25 µl H_2O and 20 µl PCR ready-to-use mixture (HotStart-PCR Kit, Qiagen, Hilden, Germany) in 35 thermal cycles of 95, 60 and 72°C for 45, 60 and 90 s, respectively (MiniCycler Bio-Rad, CA, USA). The PCR products were purified by using a column based cDNA clean up system (MiniElute PCR Purification Kit, Qiagen, Hilden, Germany). The sequence determinations of the PCR products were carried out by AGOWA DNA sequencing service (AGOWA, Berlin, Germany). The cDNA sequences were translated to the predicted amino acid sequence by using the DNAsis program (MedProbe, Oslo, Norway). The amino acid sequence was then compared with sequences from the non-treated control and wild-type, respectively as well as with sequences available from previous studies reporting of mutation induction in *rpoB* (Nicholson and Maughan, 2002; Maughan et al., 2004). Alignment of the sequences was carried out with the CLUSTAL program (EMBL-EBI, Cambridge, UK).

2.3.4 Sample requirements for studying the transcriptional profile

Samples of germinating spores after several spore treatments (i.e. UV-C and UV-A irradiation, irradiation with heavy ions or X-rays, or exposure to high vacuum) and of untreated (i.e. control) germinating spores were taken after the appropriate recovery times, centrifuged at 4°C and stored at -80°C until further analysis i.e. RNA extraction (2.1.8) for cDNA labeling (2.4.2) as principal component for transcriptional profiling *via* DNA microarray technology as described in chapter 2.4.

2.4 Gene expression analysis via cDNA microarray technology

Several molecular biology techniques have been developed over the past 40 years to analyze the functions of the genome. It started in 1975, when Edwin Mellor Southern showed that immobilized single-stranded (ss) DNA molecules can hybridize with their complementary sequences (since then this method is termed "Southern blot") (Southern, 1975). In a normal Southern blot target ssDNA population is immobilized on a membrane and a labeled probe is hybridized to it. In a further development of this dot-blot system, DNA arrays can now be prepared on glass or silicon substrates in a much higher number and density of spots (= number of genes). The DNA spots that constitute a DNA array can be composed of short oligonucleotides (25 - 75 bp), partial gene sequences (75 - 500 bp), or full-length cDNAs. DNA arrays are usually printed in miniature with polymerase chain reaction (PCR) products or oligonucleotides on glass microscope slides (microarrays), or in larger formats on membranes (macroarrays) (Rockett and Dix, 2000). The experimental design must allow for true steady-state conditions or include a sampling interval that appropriately reflects the timing of genetic responses and, in both cases, avoid the introduction of artifacts caused by poor sampling technique. Microarray expression profiling is now a universal tool, with a wide range of applications that benefit from accurate determination of differential gene expressions (Lucchini et al., 2001; Ye et al., 2001).

In this thesis, the transcriptional response during germination of treated (i.e. irradiated or vacuum-exposed) samples was compared to those of identical, but untreated ones (i.e. non-irradiated or non-vacuum-exposed control). This comparison is the most reasonable strategy for measuring differential gene expression resulting from exposure to damaging agents. The identification of genes and pathways by these expression profiling studies was complemented by monitoring physiological or genetic

responses, i.e. survival or mutation induction, and characterization of DNA lesions (induction and repair). This experimental strategy of "splitting a culture" before treatments (e.g. irradiated versus non-irradiated) and similar treatment (i.e. recovery by incubation) after treatment avoids inadvertent artificial changes in gene expression. For comparison of deficient strains with wild-type strains, irradiated mutants were compared with non-irradiated deficient cells from the same batch (Conway and Schoolnik, 2003).

A prerequisite for such gene expression studies is the availability of the complete genome sequence of that organism. Since the first sequencing of a whole bacterial genome (*Haemophilus influenzae* R1; Fleischmann et al., 1995), genomes of more than 630 organisms have been sequenced (http://www.genomenewsnetwork.org/resources/sequenced_genomes/genome_guide_index.shtml) and > 2870 more are currently under investigation (http://www.genomesonline.org) (status at August 13rd, 2007). The completion of the *B. subtilis* genome sequence (strain 168) was first announced on July 19th, 1997 during the 9th International Conference on *Bacilli* in Lausanne, Switzerland and then published on November 20th, 1997 in Nature: F. Kunst, N. Ogasawara, I. Moszer, <146 other authors>, H. Yoshikawa, A. Danchin "The complete genome sequence of the Gram-positive bacterium *Bacillus subtilis*". A total of 4107 putative ORFs (open reading frames) were assigned to the *B. subtilis* 168 (Bsu) genome (Kunst et al., 1997).

2.4.1 Microarray fabrication and construction

Microarray construction was based on the genomic sequence data and annotation provide by the *B. subtilis* genome sequencing project (http://genolist.pasteur.fr/SubtiList/help/project.html).
For manufacturing whole-genome containing glass cDNA microarrays a *B. subtilis*-OligoLibrary was obtained from Sigma-Genosys (Sigma-Genosys, Haverhill, UK). It contains the complete collection of oligonucleotides designed to optimally represent all *B. subtilis* genes found in the genome data release R16.1 on the SubtiList website (http://genolist.pasteur.fr/SubtiList/). Each gene was represented by a single oligonucelotide with a specific length of 65 bp.

The complete *B. subtilis*-OligoLibrary, transferred in 384-well plates, was mechanically spotted with the MicroGrid II - System (BioRobotics, Oberhaching, Germany) on chemically modified glass slides

(Greiner Bio-One GmbH, Nuertingen, Germany) in the DNA-microarray-core-facility at Max-Planck-Institute for Infection Biology in Berlin, Germany (in cooperation and collaboration with Dr. H.-J. Mollenkopf). The array geometry was partitioned in 8×4 subgrids (each subgrid contained 130 spots, *B. subtilis*-Oligos and spotting buffer samples) with a spot radius of 180 µm and a spot distance of 0.3 mm (Fig. 2-9). After spotting all microarrays were dried at 80°C for at least 2 h.

Each microarray was providently chemically blocked (with a 0.1 M solution of succinic anhydride solved in 1-methyl-2-pyrrolidinone) for background saturation, UV cross-linked (UV-C: 2400 mJ/m²) to prevent subsequent binding of DNA and pre-scanned for completeness of each spotted gene before using. Microarrays were stored at 20°C in a close chamber with constant humidity at least less than 20 % absolute humidity and pure nitrogenous atmosphere until further usage.

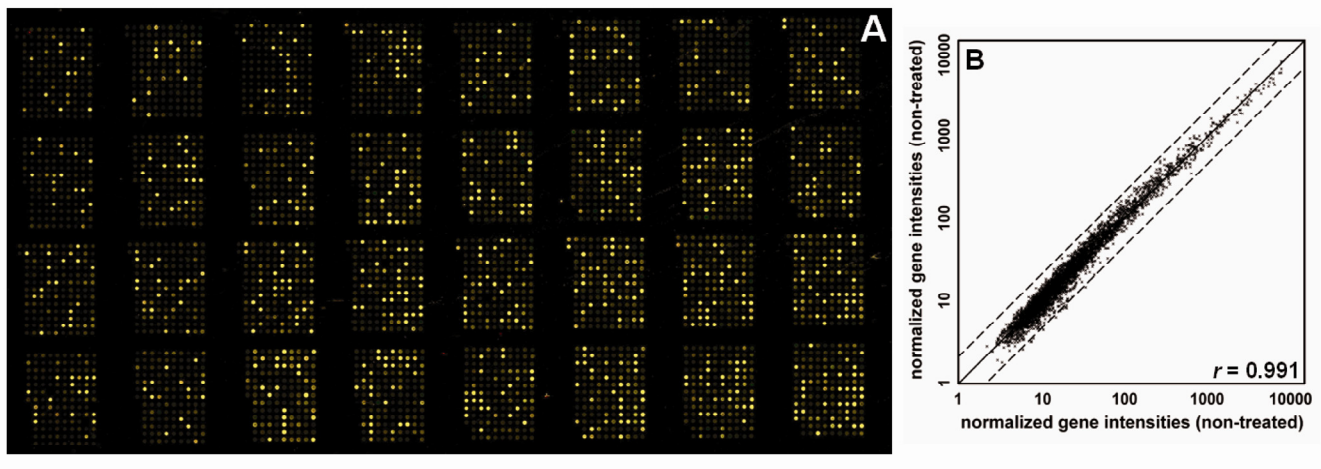


Fig. 2-9 Overview of a *B. subtilis* 168 DNA microarray design (**A**) (8×4 subgrids; experimental conditions: a *self-versus-self* hybridization; *B. subtilis* 168 (wild-type) exponential growth phase) and a scatter diagram of normalized spot intensities (**B**). Gene intensities of a *self-versus-self* hybridization from a germinating *B. subtilis* 168 culture (1 h germination, after Moeller et al., 2006). The significance threshold of twofold induction or repression is symbolized by the two dashed lines. Data are expressed as mean values (n = 3). r = Pearson correlation coefficient.

2.4.2 Preparation of fluorescent-labeled cDNA

Total RNA was extracted from germinating and outgrowing spores of *B. subtilis* by a rapid acid phenol method as described in 2.1.8. To convert the total RNA (25 µg) into labeled cDNA, reverse transcription was performed in a 0.2 ml microcentrifuge tube. Preparation of cDNA targets was based on a described method (LabelStar protocol from QIAGEN GmbH, Hilden, Germany). For probe synthesis, specific *B. subtilis* cDNA labeling primers (Sigma-Genosys, Haverhill, UK) were used for priming whereas the labeling was achieved using either the Cy3-dUTP or Cy5-dUTP fluorophore (Amersham Pharmacia Biotech, Freiburg, Germany).

After cDNA synthesis, the RNA template was removed with addition of 2.5 M NaOH and purified with a Qiagen PCR Mini kit (QIAGEN GmbH, Hilden, Germany). The labeling efficiency was checked by determining the absorbance at 260 nm for the cDNA concentration, the absorbance at 550 nm for Cy3 incorporation, and the absorbance at 650 nm for Cy5 incorporation by using a NanoDrop photometer (Kisker, Steinfurt, Germany) (Fig. 2-10).

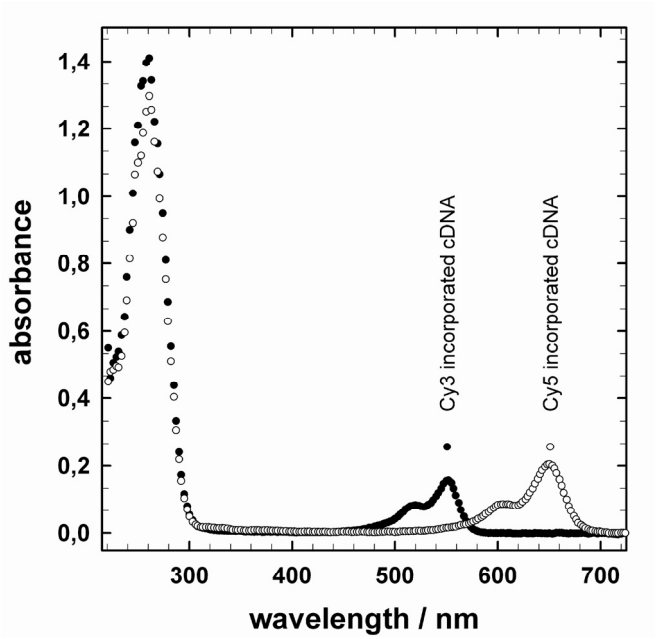


Fig. 2-10 Cy3 (λ_{max} at 550 nm) and Cy5 (λ_{max} at 650 nm) incorporation in the fluorescent labeled cDNA.

2.4.3 Microarray hybridization and washing

The control RNA samples (non-treated) and the test RNA samples (treated i.e. irradiated) were each labeled with both Cy3 and Cy5 dyes to prevent artifacts related to different labeling efficiencies. Each

single experiment required two slides; the Cy3-labeled non-treated control and the Cy5-labeled treated sample were hybridized on the first slide, and, similarly, the Cy5-labeled non-treated control and the Cy3-labeled treated sample were hybridized on the second slide, in the so-called color swap procedure. As a result, each experiment was repeated at least three times requiring six slides (each experiment was analyzed according to the color swap treatment). The use of replicates aids in distinguishing between genes that are truly differentially expressed and those affected only by noise. An equal amount of Cy3-dUTP or Cy5-dUTP-labeled probes, based on the incorporated dye concentration, was applied to each slide. The labeled cDNA was concentrated to 10 µl (total volume) and mixed with 35 µl hybridization buffer (SildeHyb Glas Array Hybridization Buffer 1; Ambion, Cambridgeshire, UK). The hybridization was carried out at 42°C for 18 h with microarray slide hybridization buffer containing formamide (Ambion, Huntingdon, UK). Slides were washed at 15 min intervals, once with a solution containing 2×SSC (1×SSC is 0.15 M sodium chloride NaCl (Merck KGaA, Darmstadt, Germany) plus 15 mM sodium citrate (Merck KGaA, Darmstadt, Germany) and 0.1 % sodium dodecyl sulfate (SDS; Merck KGaA, Darmstadt, Germany) at 37°C and three times with a solution containing 0.1×SSC and 0.1 % SDS at 42°C. The slides were then rinsed with 0.1×SSC and distilled water. After drying under a stream of N_2 and/or dry spinning via centrifugation, the slides were scanned for the fluorescent intensity of both the Cy5 and Cy3 fluorophores.

2.4.4 DNA microarray scanning and data processing

Relative mRNA levels were determined by parallel two-color hybridization to cDNA microarrays representing 4106 open reading frames (ORFs) representing 99.5 % of *B. subtilis* 168 ORFs according to Kunst et al. (1997). Scanning of microarrays was performed with 10 µm resolution using a DNA microarray laser scanner (DNA Microarray Scanner BA, Agilent Technologies, Agilent, Böblingen, Germany). Features were extracted from the raw image data generated by laser scanning, using the Agilent Technologies image analysis software (G2567AA Feature Extraction (FE) Software, Version A7.5). In a first step, the microarray TIFF-files were flipped upper left to lower right and were saved as modified images. The microarray grid was set up interactively using a gal file generated by TAS Application Suite (Biorobotics, MicroGrid II) for 32 blocks according to GenePix ArrayList V1.0 (Molecular Devices Corporation, CA, USA). The gal file was converted into Microsoft excel to reversed orientation. The array geometry including main grid, subgrid, and nominal spot diameter was

adjusted using the preset values of the FE software and the autofit modus. The identified array geometry was checked and refined manually if required. For each scanned hybridization an individual grid file was generated and the grid files were stored as comma/character separated values (CSV). CSV files were used for feature extraction with the following FE configuration: spot deviation limit 250 µm, calculated spot size and cookie cutter method. Pixel outlier rejection, poly outlier flagging including non-uniformity and population outlier flagging was used with software defined values. The local background subtraction method was used and the background globally adjusted to zero. Dye normalization for removal of systematic differences in dye bias was done by selecting normalization features with the rank consistency method together by applying a linear normalization (Smyth and Speed, 2003) across the entire range of data together with a non-linear normalization (linear and LOWESS). The ratio between both channels, the log ratio error and the p-value as measures of differential expression was calculated using the most conservative estimate of errors between universal error model and propagated error and standard multiplicative and additive error settings together with the use of surrogates. Feature extraction results were stored in GEML, MAGE-ML and tab-delimited text version together with JPEG image file and visual results. The tab-delimited files of the individual hybridizations were used for further analysis. The user manual and detailed information on the way FE software calculates results can be retrieved at the Agilent (Agilent Technologies, Waldbronn, Germany) Technology website (http://chem.agilent.com). Additional, to the standard used image analysis software "Feature Extraction" (Agilent), the acquired TIFF-images were parallel analyzed and aligned by using ScanAlyze software, which is publicly available at http://rana.stanfort.edu/software and by data evaluation with the software package "ImaGene" (BioDiscovery Inc., Bemmel, Netherlands; www.biodiscovery.com/index/imagene).

Ratios were calculated with averages of the parallel samples (from biological (independent experiments) and technical (hybridized as replicates and/or color swaps) duplicates; according to Eichenberger et al., 2003; Au et al., 2005). Genes were identified as differentially expressed (i.e., induced or repressed) if the expression ratio (ratio of the normed mean Cy3 to the normed mean Cy5) was greater than twofold and the *P* value (as determined by a *t*-test) was less than 0.005 (according to the data analysis used by Smyth and Speed, 2003; Steil et al., 2003). Genes that showed a statistically significant difference in expression ($P < 0.005$) and were > 2-fold in magnitude as up-regulated and <

1.5-fold as down-regulated were analyzed further. *P* values were calculated with log-transformed, normalized intensities. Normalization was relative to the median total fluorescence intensity per slide per channel. The gene functions were obtained from the databases at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) and the SubtiList World-Wide Web Server (http://genolist.pasteur.fr/SubtiList/) (Moszer et al., 2002).

In this work, gene expression for a particular comparison of conditions was considered to be changed when three criteria were fulfilled: (i) expression of the gene had exceeded the background signal level, (ii) changes in expression of the gene had to be statistically significant as defined in a group comparison of the values for the selected conditions with a nonparametric test (Wilcoxon's test; Scheer et al., 2006) and a Benjamini and Hochberg false discovery rate correction (Scheer et al., 2006 and described below), and (iii) the change in expression had to exceed a factor of 2.5, to be listed.

2.4.5 Statistical significance data analysis of differential gene expression

DNA microarrays contain oligonucleotide or cDNA probes for measuring the expression of thousand of genes in a single hybridization experiment. Because massive amounts of data are generated, methods are needed to determine whether changes in gene expression are experimentally significant. The type of experiment directly affects the downstream analysis of the data. Standard *t*-tests (i.e. Welch's *t*-test) were used for two-group comparisons of experimental conditions (Welch, 1938, Olson, 2006). After identification of genes with a differential expression, separations of genes with a specific regulation pattern were performed (i.e. clustering). Cluster analysis of microarray experiments allowed determining coherent patterns of gene expression (partition clustering give the expression profile of the experimental design). Common methods based on conventional *t*-tests provide the probability (*P*) that a difference in gene expression occurred by chance. The gene expression ratios of the treated samples to the respective untreated control were normalized using the pooled-common-error model provided by the statistical analysis software ArrayStat v. 2.0 (Imaging Research Inc., Ontario, Canada). Parallel to the chosen confidence level (*P* value) the rate of false discovery (FDR) was observed (Li et al., 2006). Specific statistical methods were developed for microarray analysis e.g. SAM Significance Analysis of Microarrays (Tusher et al., 2001). SAM identifies genes with statistically significant changes in expression by assimilating a set of gene-specific *t*-tests. Each gene is assigned to a score on the basis of

its change in gene expression relative to the standard deviation of repeated measurements for that gene. Genes with scores greater than a threshold are deemed potentially significant. All microarrays / hybridizations were statistically analyzed by using the software "Significance Analysis of Microarrays". SAM is publicly available at http://db.systemsbiology.net/software/VERAandSAM. Non-commercial and commercial software packages are available for microarray data analysis and in most instances, large sets of statistical approaches and applications (i.e. normalization, scatter plotting, ratio-histograms, background correction, threshold predefinition, *t*-test, significance analysis of gene groups, clustering and several more features) are serially integrated and were standard used in this work (strengthening through the usage of several different software applications and tools). The determination of the (biological) statistical significance of gathered microarray data is one of the greatest challenges in the bioinformatic field of studying differential gene expression.

2.4.6 Microarray data interpretation

Once a list of differentially expressed genes has been generated, the next task is to determine the biological significance of the genes in that list. By combining the identification of broad biological themes with the ability to focus on a particular gene, it is possible to characterize the biological function the gene belongs to and to identify particular genes of interest from a large list of potential targets.

Combined methods, i.e. database mining and literature search, were used to test the robustness of the microarray (hybridization) data as follows:

(i) the direct correlation of gene expression with the predicted operon organization by data alignment with common databases such as NCBI-PubMed and SubtiList. Operons are the principal form of gene coregulation in prokaryotes; therefore the expression patterns of genes within an operon are expected to be correlated (Karlin and Marzek, 2000; Korbel et al., 2004). For this purpose, the DBTBS database (http:dbtbs.hgc.jp/), a collection of experimentally validated gene regulatory relations (network characterization), was used (Ishii et al., 2001; Makita et al., 2004).

(ii) the intercomparison of up-regulated yet unknown genes with those of known functions in other (close related) bacterial systems by using the Smith-Waterman report. The Smith-Waterman report is a data search/alignment against a non-redundant protein databank ("Nrport") with a specific *B. subtilis*

168 protein sequence as the query. Nrport is made up of a combination of SWISS-PROT (http://www.expasy.org/sprot/), GenePept (NCBI-PubMed), PIR (http://pir.georgetown.edu/) and NRL3D (htttp://www.infobiogen.fr/db/NRL3D/). Smith-Waterman searches for all *B. subtilis* 168 proteins are performed on aquad-processor Pracel GeneMatcher (updated on a regular basis, monthly).

(iii) additional data alignment to gene ontology with the databases PRODORIC (http://prodoric.tu-bs.de/) (Muench et al., 2003), AmiGO (http://amigo.geneontology.org/cgi-bin/amigo/go.cgi) (Liu et al., 2001) and KEGG, the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) (Kanehisa and Goto, 2000) were carried out to study the function, homology and regulation of the selected gene(s).

(iv) the relation of the expected DNA damage, e.g., by UV radiation, to the known activity of the up-regulated gene e.g. *uvrABC* involvement, as can obtained from the functional interpretation of the transcriptional activities using the JProGo website (http://www.jprogo.de) on the basis of gene ontology (Scheer et al., 2006).

(v) independent mRNA quantification of selected candidate genes from treated and untreated samples (method described in 2.4.7).

Current research shows that combining both, literature search and microarray analysis, which share the common goal of identifying the "hidden" networks of biological entities (Li et al., 2006; Liu et al., 2005), are one of the suggestive multivariate selection procedures (Fig. 2-11). Integrating both, experimental data and the literature knowledge in an iterative fashion is an effective way in biological network modeling (according to Le Phillip et al., 2004). This approach was used as "guidepost" for the microarray data analysis, particularly for determining the biological significance of a discovered gene regulation (Goodman, 2002).

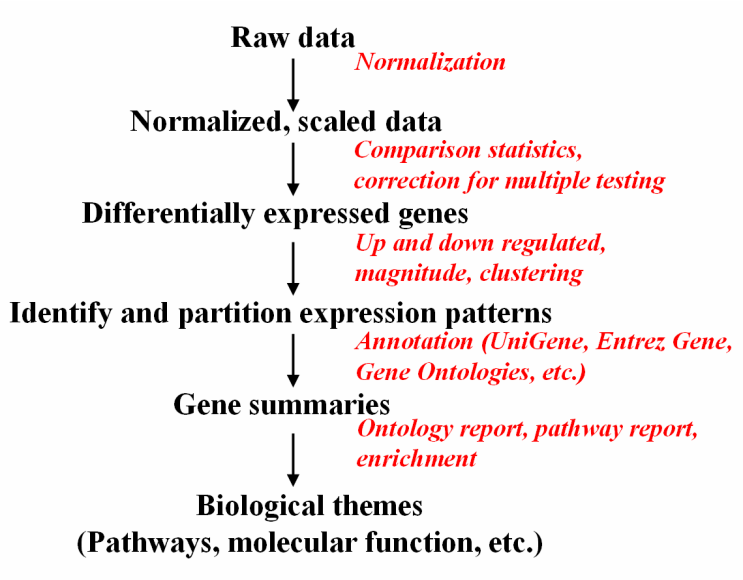


Fig. 2-11 The overall goal of the microarray data analysis process is to take raw gene expression data and identify the biological significance of this gathered data. This figure shows the general workflow studying differential gene expression via array technology, the specific methods used at each step depend on experimental factors such as experiment design, array image analysis, database mining and gene integration in systematical approach (Olson, 2006)

2.4.7 Comparative evaluation of the mRNA pool via quantitative RT-PCR

DNA probes for selected candidate genes (particularly "*y*-genes", which by database(s) mining are suggested to be involved in DNA repair processes) were synthesized by using a One-Step-RT-PCR-Synthesis kit (SuperScript III kit from Invitrogen, Karlsruhe, Germany) and gene-specific arranged primers (MWG, Ebersberg, Germany). The length of the synthesized probes was in the range of 230 - 270 bp. Total RNA (approx. 100-200 ng), from independent experiments (which were different from those used to harvest RNA for the DNA microarray analyses), was reverse transcribed and amplified in a single tube in a 50 µl volume consisting of 25 µl 2 × reaction mix (containing dNTPs in buffered solution), each 1 µl of forward and reverse primer (all primer sequences used in this work are listed in Table 2-7), 1 µl RT-Taq polymerase (QIAGEN GmbH, Hilden, Germany) and 20 µl of RNase free H_2O (Gibco BRL, Life Technologies, Karlsruhe, Germany). The reaction mixture was subjected to reverse transcription at 50°C for 30 min followed by 35 cycles of rapid PCR amplification (PCR conditions: denaturation at 94°C for 15 s, annealing at 57°C for 30 s, and extension at 70°C for 60 s) with a final extension unique cycle at 72°C for 10 min. The reverse transcribed cDNA, of both experimental conditions (treated, untreated), was evaluated by gel electrophoresis (as described in detail by Nellen et al., 2006). 3 µl of each cDNA sample was transferred on a 1.5 % (w/v) agarose gel (Serva, Heidelberg, Germany), containing ethidium bromide (4 µg/ml) with 3 µl of a standard DNA

marker for 100 bp up to 2 kb product size (Hyper Ladder I and IV, Bioline GmbH, Luckenwalde, Germany).

Table 2-6 Used primers and their respective sequences

Gene	Primer direction	Sequence
uvrX	forward	5'-AGGAGGGAGGATGGAAAGAA-3'
	reverse	5'-TCTCCAGAAGAACCGCCTTA-3'
yefB	forward	5'-GCCACGAACCAATCATACCT-3'
	reverse	5'-GCCACGAACCAATCATACCT-3'
yefC	forward	5'-AAGCTGCGAGGGACTAGTGA-3'
	reverse	5'-CAAATTCAGCAAACGCAGAG-3'
yhaZ	forward	5'-ACATTACGGCTGGAGGACTG-3'
	reverse	5'-GGGGCTGTATCCCGTAAAAT-3'
yjcD	forward	5'-GCCAGTCCAGAAGACTTTGC-3'
	reverse	5'-GTGTTTAATCGCCTCGGTGT-3'
yqjH	forward	5'-GCTTCAGCGAGTGTTCATGC-3'
	reverse	5'-GCTTCAGCGAGTGTTCATCA-3'
ykoQ	forward	5'-TCCGAAACTGGCAGGTTACT-3'
	reverse	5'-CGTGATACAGCCGTTTTCCT-3'
ymaD	forward	5'-ATGTCGGTACGATCGAAAGC-3'
	reverse	5'-GCATTTCATCTGGGTTGGTC-3'
yneB	forward	5'-TATGCCCGTGTAAGCACAAA-3'
	reverse	5'-CGAGCAGCTCAAATACACCA-3'
yobH	forward	5'-TGTGCACAGTTGGAATTGGT-3'
	reverse	5'-TTCTTTCCATCCTCCCTCCT-3'
ywjD	forward	5'-CTCTGTTTACGAGCCCGAAG-3'
	reverse	5'-ACAAACGGAACATCCTCCTG-3'

2.5 Numerical and statistical analysis

For the determination of the survival fraction of treated spores the ratio of N (= colony forming units with treatment) and N_0 (= colony forming units without treatment) was calculated. The survival was semi-logarithmically plotted as function of the respective treatment (fluence, dose, or time).

n = number of experiments (number of samples at e.g. selected fluence),

N = mean values of the treated colony forming units,

N_0 = mean values of the colony forming units without treatment (control),

S = survival fraction (ratio of treated to untreated) $= \left(\frac{N}{N_0} \right) = S$,

s = standard deviation of (N),

ΔN = standard error of (N),

ΔS = standard error of (S) at treatment X (e.g. UV-C irradiation),

For the determination of the standard error ΔS of S following equation was used:

$$\Delta S = S \times \sqrt{\left(\frac{\Delta N}{N} \right)^2 + \left(\frac{\Delta N_0}{N_0} \right)^2} \qquad \textit{(Eq. 2)}$$

with $\Delta N = \frac{s(N)}{\sqrt{n}}$.

Mutation induction curves were obtained by plotting the mutation frequency versus the dose (for X-rays and accelerated heavy ions) or exposure days (for high vacuum storage). Induced mutation frequencies were measured and calculated according to Baltschukat and Horneck (1991) using:

$$M = \frac{(m - m_0)}{N} \qquad \textit{(Eq. 3)}$$

M = mutation frequency,

m = colony formers carrying a induced mutation (after treatment),

m_0 = colony formers carrying a spontaneous mutation (without treatment),

N = total colony formers (after treatment).

All experiments were repeated at least three times and the data shown are mean values with standard deviation. All inactivation, germination and repair curves of treated spores were compared statistically using Student's *t*-test. Differences with P values ≤ 0.05 were considered as statistically significant.

2.5.1 Survival curve analysis

Cell survival, in radiobiological terms, is understood as the ability for indefinite reproduction. The principle of the basic technique to measure cell reproduction is very simple: treated cells are brought on a suitable nutrient medium solidified by agar, in such a density (i.e. diluted) that they are able to divide without interfering with each other. On incubation, each cell which did not lose its reproductive

integrity will grow to a visible colony, the so called colony formation ability (Puck and Marcus, 1956). The number of dilution steps has to be appropriately adjusted to the expected survival rate. In biological terms, a long time between the initial insult and the ultimate effect during which many modifying processes may intervene. Irradiated cells do not immediately lose its division ability, a dose depending effect. Colony formers are usually called "survivors". If their fraction is plotted versus dose, one speaks of "survival curves". It is customary in radiation biology to plot the surviving fraction logarithmically on the ordinate versus dose on a linear abscissa scale. Two representatives of survival curves are shown in Fig. 2-12.

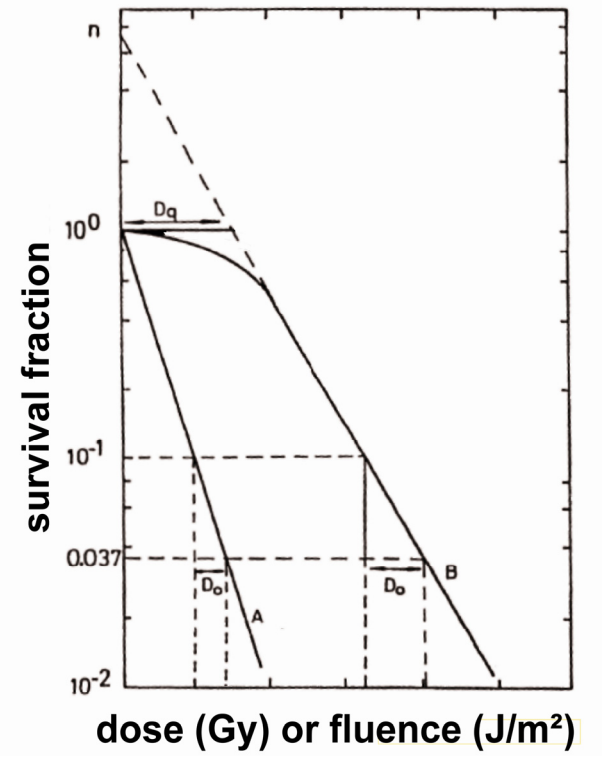


Fig. 2-12 Different types of cell survival curves after radiation exposure to UV and ionizing radiation.

Homogeneous cell populations display generally one of the types of survival curves after irradiation as shown in Fig. 2-12. Example (A) is the simplest case: Since the semi-logarithmic plot yields a straight line, the functional relationship is a simple exponential one. In example (B), the straight line is only approached with higher doses, while in the lower region a threshold-like behavior is seen. For microorganisms curve (B) is mostly the usual case. It is the slope of the terminal straight part which is commonly indicated by the dose – termed "D_0" – which is necessary to reduce relative survival to e^{-1}

(0.37). D_0 is obviously the reciprocal of the slope. There are two ways to deal with the initial part both making use of the backward extrapolation of the straight portion. Its intercept with the ordinate is called "extrapolation number" (n), the dose where it crosses the 100 %-survival line is termed the "quasi-threshold dose" D_q.

$D_0 = \frac{D_q}{\ln n}$, is a dependent correlation of all three parameter. D_0 is the most important parameter to characterize the sensitivity of a cellular system. The term of "dose" is basically used for ionizing radiation i.e. X-ray and "energy fluence" (fluence) is used for UV. Dose, in the strict sense of the word, means absorbed energy per unit mass and can be used only with ionizing radiation. With UV radiation, this occurs via selective absorption in the chromophores while for ionizing radiations, all constituents of the exposed system contribute (Hutchinson, 1985).

The method for determining the colony formation ability (index for survivability) is described in detail in chapter 2.1 (under 2.1.5). Survival was determined from appropriate dilutions in distilled water as colony forming ability (CFA) after growth overnight on nutrient broth agar (Difco Detroit, MI, USA) at 37°C. To determine the characteristic curve parameters after UV radiation (Table 2-7), the following relationship was used: $\ln\left(\frac{N}{N_0}\right) = -k_i \times F + n$ within N = colony formers after UV-irradiation; N_0 = colony formers without UV-irradiation; IC = inactivation constant (m^2/J); n = extrapolation number, i.e. the intercept with the ordinate of the extrapolated semi-log straight-line.

The constants k_i and n were determined by linear regression. The ratio of the k_i values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). It reflects the contribution of the involved repair processes to overall survival. Further, the fluence/dose resulting in 10 % survival after irradiation (F_{10} or D_{10}) was determined. The inactivation constant was determined from the slope of the 'fluence/dose'-effect-curves.

Table 2-7 Summary of the analyzed characteristic curve parameters

Parameter	Definition
D/F_{10}-value (Gy / J/m²)	dose/fluence reducing the survival to 10 %
D/F_0-value (Gy / J/m²)	reduce relative survival to e^{-1} (37 % survival)
D/F_q-value (Gy / J/m²)	quasi-threshold dose/fluence
IC (Gy^{-1} / m²/J)	inactivation constant
n	extrapolation number i.e. the intercept with the ordinate of the extrapolated semi-log straight-line
R^2	regression coefficient defined as the slope of the equation that most closely relates the correlated variables (quality of the regression relationship)

2.5.2 Numerical analysis of the DNA photoproduct status

Photoproduct induction curves were obtained by plotting the number of photolesions per 10^4 bases versus fluence. The efficiency of photoproduct induction was determined from: $PP = k_{PP} \times F$, with PP = photoproducts/10^4 bases, k_{PP} = photoproduct induction constant (m²/J) and k_{PP} was determined by linear regression of the induction curves. For quantifying the distribution of the UV-induced DNA bipyrimidine photoproducts in the irradiated spores, data from each fluence were summarized, normalized and expressed in number of photolesions/10^4 bases per Jm^{-2} according to Pogoda de la Vega et al. (2005) and Moeller et al. (2007a; 2007b).

All experiments (*n* = number of the experiments) were repeated at least three times and the data shown are mean values with standard deviation. The survival data and photoproduct analysis curves of the irradiated colony forming units were compared statistically using Student's *t*-test (Welch, 1938). Differences with P values ≤ 0.05 were considered statistically significant. With the aim of comparing the resistance of spores of *B. subtilis* with different genetic backgrounds, the vegetative cells and (dormant, germinating and outgrowing) spores were all prepared at the same time and tested together.

3. Results

It is the aim of this study firstly to characterize damage induced by external stressors in the DNA of dormant spores of *Bacillus subtilis* 168. The stressors selected are UV radiations of different wavelength ranges that cause specific damage to specific bases of the DNA, ionizing radiations, of low and high LET producing mainly breakage of DNA strands, extreme desiccation by vacuum treatment leading to loss of most cellular water, and high shock pressures combined with high peak temperature increases affecting also spore components other than DNA. Secondly it is aimed at determining the role of different DNA repair strategies in the recovery of the treated spores during germination and outgrowth. These repair systems may act damage-specific, such as the SP repair system, or more damage-unspecific, such as recombination-mediated repair and probably several not yet identified systems. Special emphasis was laid on the identification of DNA-damage-responsive genes and their possible roles in cellular repair and recovery processes. This was based on global transcriptional responses to sublethally damaging treatments that were determined for each stressor.

To determine the role of different repair systems in the recovery processes, the responses of spores of known genetic characteristics in DNA repair capability were compared. They include a wide spectrum of strains with regard to DNA repair, i.e. wild-type as well as deficient strains in either universal or spore-specific DNA-repair (Table 2-1, 2.1.2). To obtain comparable results, all specimens were treated and analyzed under identical conditions.

3.1 Setting up the experiment protocol: spore germination and RNA isolation

3.1.1 Kinetics of spore germination of different *Bacillus* sp. strains

It is notable that the genetic outfit of spores as well as their treatment with different stressors may affect the subsequent germination kinetics. A typical profile of spore germination of the wild-type strain is shown in Fig. 3-1. These spores were not treated by any stressor. Characteristic features are (i) the decrease in OD during the first 30-60 min of incubation, caused by the excretion of spore components, such as Ca-DPA, and by spore core hydration (Setlow and Setlow, 1996; Setlow, 2003), which is then followed by an increase in OD as a consequence of spore outgrowth and subsequent cellular growth;

(ii) the gradual loss of heat resistance, measured as decrease in CFA of heat-treated samples, reflecting the progressive loss of spore integrity; and (iii) an increase of CFA after about 90 min of incubation indicating the initiation of replication and subsequent cell division. The loss of heat resistance was detectable already shortly after initiating spore germination and declined by 2 orders of magnitude after 60 min of incubation. These germination profiles of non-treated spores agree well with published data on spore germination and outgrowth of *B. subtilis* (Setlow and Setlow, 1996).

Comparison of the germination profiles of non-treated spores with those of UV irradiated (125 J/m² UV-C radiation) spores (Fig. 3-1) did not give any statistical significant difference concerning CFU counts and decrease in heat resistance. This UV dose caused an inact vation by 1 order of magnitude in wild-type spores. A significant increase in the total CFU was measured after 120 min for both, non-treated as well as UV-irradiated spores. However, the delayed increase in OD values of UV-irradiated spores indicates an extended lag-phase of outgrowth after UV irradiation. Concering the other tested stress parameters, i.e. sub-lethal doses of UV-A, X-rays, accelerated heavy ions or exposure to high vacuum, the germination profiles did not show any significant differences with those of untreated spores.

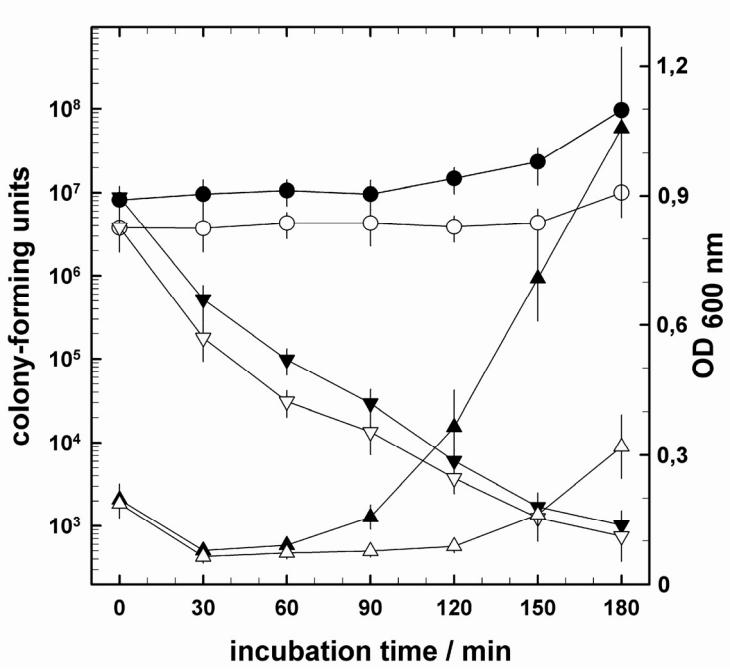


Fig. 3-1 Germination profile of *B. subtilis* 168 spores (solid symbols) examplifying the physiological changes in dormant, germinating and outgrowing spores. Viable (circle) or heat resistant (triangle down) cells were determined from CFU counts, OD readings at 600 nm (triangle up). For comparison the germination profile of *B. subtilis* 168 spores irradiated at 125 J/m² UV-C (254 nm) (open symbols) is shown. Data are averages +/- standard deviation (n = 5).

Examples of typical spore germination profiles are given in Fig. 3-1 (after UV-C irradiation) and Fig. 3-2 (after chemical treatment). Based on these data and in agreement with previously published data by Setlow and Setlow (1996) a standard experimental protocol for studying DNA damage and repair in germinating and outgrowing spores was set up and used in all further investigation as follows:

After spores were exposed to the different stress conditions, first dose-effect curves for survival were determined. Then spores were incubated and samples were taken at different intervals (dormant spores at 0 min of incubation, from germinating spores at 30 and 60 min of incubation, and from outgrowing spores at 90 and 120 min of incubation) for DNA extraction and DNA damage analysis. For studying global gene expression, RNA was isolated from samples taken at the same intervals; however, only one dose was applied that inactivate spores by approximately one order of magnitude (± standard deviations).

Table 3-1 Germination efficiency[a] of non-treated and of UV-C irradiated spores of wild-type *B. subtilis*; data relative to those of spores before germnation

A. non-treated spores

Germination time / min	Total CFU	Heat-resistance	Turbidity / $OD_{600\ nm}$
0	1.00 ± 0.13	1.00 ± 0.09	1.00 ± 0.12
30	1.17 ± 0.09	$(5.91 \pm 1.58)\times10^{-2}$	0.39 ± 0.06
60	1.29 ± 0.16	$(1.09 \pm 0.22)\times10^{-2}$	0.46 ± 0.10
90	1.17 ± 0.06	$(3.23 \pm 0.76)\times10^{-3}$	0.79 ± 0.14
120	1.80 ± 0.20	$(6.89 \pm 1.02)\times10^{-4}$	1.85 ± 0.23
150	2.87 ± 0.41	$(1.93 \pm 0.54)\times10^{-4}$	3.60 ± 0.33
180	11.83 ± 3.22	$(1.16 \pm 0.30)\times10^{-4}$	5.38 ± 0.49

B. UV-C (125 J/m²) irradiated spores

Germination time / min	Total CFU	Heat-resistance	Turbidity / $OD_{600\ nm}$
0	1.00 ± 0.17	1.00 ± 0.10	1.00 ± 0.04
30	0.98 ± 0.12	$(4.72 \pm 0.77)\times10^{-2}$	0.35 ± 0.05
60	1.14 ± 0.09	$(8.13 \pm 2.39)\times10^{-3}$	0.39 ± 0.08
90	1.16 ± 0.15	$(3.55 \pm 0.60)\times10^{-3}$	0.41 ± 0.07
120	1.27 ± 0.19	$(9.87 \pm 2.15)\times10^{-4}$	0.53 ± 0.08
150	1.34 ± 0.21	$(3.29 \pm 0.62)\times10^{-4}$	0.94 ± 0.07
180	2.79 ± 0.43	$(1.97 \pm 0.26)\times10^{-4}$	1.85 ± 0.20

Data are expressed as averages and standard deviations (n = 4).

3.1.2 Quality control of the standard experimental protocol

In all experiments of this work, identical procedures were applied for the generation of *Bacillus* sp. spores and the further damage, repair and genome analyses. *Bacillus* sp. spores were obtained from cells cultured in a defined medium (2.1.3 and 2.1.4). This sporulation medium was selected on the basis of previous observations that spores generated were homogenous in their outgrowth and thermal resistance properties (Tables 3-1 and 3-2). After harvesting and extensive washing in ultra-pure distilled water (Gibco BRL, Life Technologies, Karlsruhe, Germany), purified spores were inspected by phase-contrast microscopy and shown to be free (≥ 98 %) of vegetative cells and any kind of cell debris. For inducing DNA damage in dormant spores, different types of *B. subtilis* 168 spores were irradiated as aqueous spore suspension with 254 nm-UV-C radiation (2.2.3.1). Only spore suspensions with a homogeneity ≥ 95 % were taken for DNA repair experiments. Immediately, after treatment, e.g. UV-C exposure at 125 J/m^2, irradiated and non-irradiated spores were transferred in germination medium at incubated at 37°C under optimal aeration. Spore germination was triggered by the supplement of *L*-alanine, as described previously. Spore germination was followed by monitoring of the optical density, total cell count and cell count after heat treatment. In Table 3-2 the total cell count and the remaining heat-resistant cells after 0 and 60 min germination are shown. After 1 h incubation in germination medium nearly two orders of magnitude were inactivated after heat-treatment independently of their genetic makeup (2.1.6).

Table 3-2 Colony formation of total and heat-resistant spores during spore germination

Strain	CFU/ml at 0 min[a]	Heat-resistant CFU/ml at 0 min[b]	CFU/ml at 60 min[a]	Heat-resistant CFU/ml at 60 min[b]
B. atrophaeus	$(1.4 \pm 0.4)\times10^7$	$(1.5 \pm 0.6)\times10^7$	$(1.6 \pm 0.3)\times10^7$	$(3.9 \pm 1.8)\times10^5$
B. subtilis (wild-type)	$(1.3 \pm 0.2)\times10^7$	$(1.5 \pm 0.4)\times10^7$	$(1.5 \pm 0.6)\times10^7$	$(0.9 \pm 0.6)\times10^5$
sspA sspB	$(2.1 \pm 0.6)\times10^6$	$(2.0 \pm 0.8)\times10^6$	$(1.9 \pm 0.5)\times10^6$	$(5.8 \pm 3.2)\times10^4$
recA	$(5.6 \pm 1.1)\times10^6$	$(5.9 \pm 0.9)\times10^6$	$(6.6 \pm 0.9)\times10^6$	$(9.8 \pm 4.1)\times10^4$
splB uvrB	$(1.0 \pm 0.5)\times10^6$	$(0.8 \pm 0.3)\times10^6$	$(0.8 \pm 0.3)\times10^6$	$(1.8 \pm 0.7)\times10^4$
recA splB uvrB	$(4.3 \pm 0.8)\times10^5$	$(4.5 \pm 1.6)\times10^5$	$(5.2 \pm 1.6)\times10^5$	$(9.2 \pm 3.0)\times10^3$

[a]Incubation was performed at 37°C in germination medium (CFU/ml) (after Moeller et al., 2006). [b]Cells and spores, surviving heat shock treatment at 80°C for 10 min were recorded as heat-resistant CFU (CFU/ml). Data are expressed as averages and standard deviations ($n = 4$).

Data on the thermal inactivation, measurements of the optical density (i.e. the release of dipicolinic acid DPA) and the microscopic inspection of the germinating cells (i.e. transition from phase-bright spores to phase-dark germinating cells) were used for analyzing the synchronization of the germination of the treated samples and the untreated control.

3.1.3 RNA isolation from dormant, germinating and outgrowing *B. subtilis* spores

The significance and reproducibility of DNA microarray experiments depend critically on the quality and purity of RNA extracted from cells. Extracting RNA from bacteria must be done carefully to avoid its enzymatic degradation by RNAses, which are ubiquitous, stable, and difficult to remove or inactivate. So far, most microarray experiments have used RNA extracted from exponentially growing vegetative cells of *B. subtilis* only, but not from spores (amongst others: Ye et al., 2000; Petersohn et al., 2001; Au et al., 2005; Koburger et al., 2005; Steil et al., 2005).

For precise gene expression experiments it is crucial to extract high-quality RNA from dormant as well as from germinating and outgrowing spores (Liu and Slininger, 2006). Extraction of intact RNA from *Bacillus* sp. spores poses special difficulties because spores possess thick layers of highly cross-linked coat proteins, a modified peptidoglycan spore cortex, and abundant intracellular constituents, such as

the calcium chelate of dipicolinic acid (Ca-DPA) and small, acid-soluble spore proteins (SASP) as protectants of spore nucleic acids (Nicholson et al., 2000; Setlow, 2006). Problems in extraction of high-quality nucleic acid from spores can be traced to methods for spore disruption and enzymatic degradation. Spores are efficiently ruptured by physical agitation with glass beads (Nicholson and Setlow, 1990), but this technique is harsh and leads to extensive shearing of both RNA and DNA (Mason et al., 1988). Intact chromosomal and plasmid DNA have been successfully isolated from chemically decoated spores (Nicholson and Setlow, 1990).

Because chemically decoated spores have been reported to facilitate the extraction of DNA (Nicholson and Setlow, 1990) they were also expected to facilitate the isolation of RNA. In Fig. 3-2 the germination profiles of spore coat-intact (INT) and of chemically decoated (DEC) spores are shown. The germination of DEC spores was slightly delayed compared with the INT spores, and the outgrowth was less efficient as inferred from the OD values at 120 minutes (Table 3-3). The statistical comparison of the three curve types (CFU, OD, and heat resistance) did not give a significant difference between those of the INT and the DEC spores.

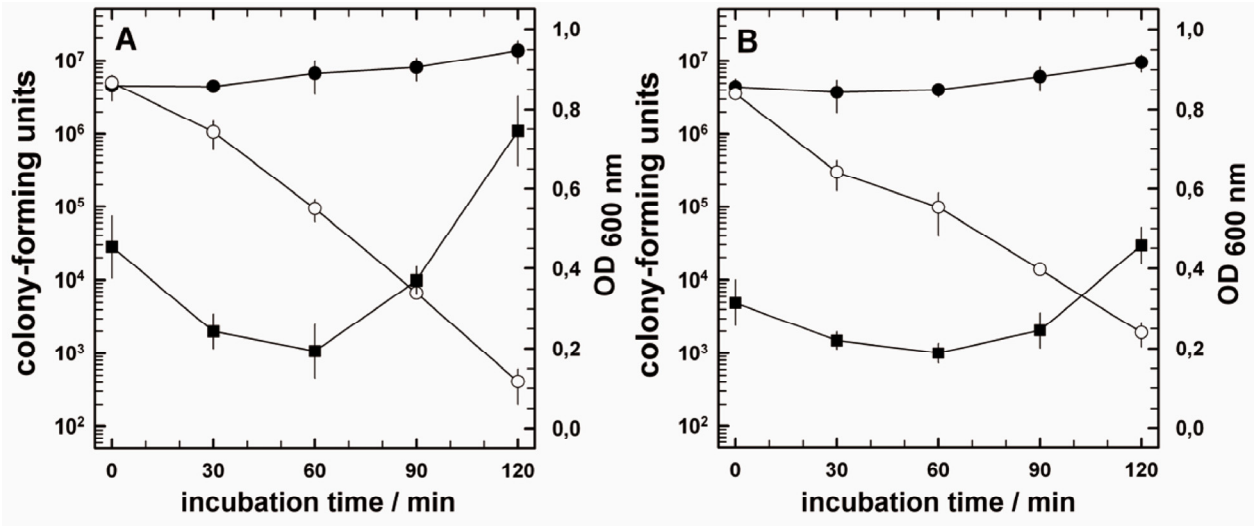


Fig. 3-2 Germination profiles of spore coat intact (**A**) and chemically decoated (**B**) *B. subtilis* 168 spores, shown as total CFUs (solid circles), CFUs after heating for 10 min at 80°C (open circles) and OD readings at 600 nm (solid squares). Data are averages +/- standard deviation (n = 4).

Table 3-3 Germination efficiency[a] of INT and DEC spores of wild-type *B. subtilis*; data relative to those of spores before germination

A. Spore coat intact *B. subtilis* spores (INT)

Germination time / min	Total CFU	Heat-resistance	Turbidity / $OD_{600\ nm}$
0	1.00 ± 0.09	1.00 ± 0.11	1.00 ± 0.07
30	0.98 ± 0.15	$(2.09 \pm 0.41)\times10^{-1}$	0.54 ± 0.14
60	1.47 ± 0.22	$(1.86 \pm 0.28)\times10^{-2}$	0.43 ± 0.09
90	1.78 ± 0.31	$(1.33 \pm 0.37)\times10^{-3}$	0.82 ± 0.20
120	2.99 ± 0.54	$(8.08 \pm 2.06)\times10^{-5}$	1.64 ± 0.57

B. Chemically-decoated irradiated *B. subtilis* spores (DEC)

Germination time / min	Total CFU	Heat-resistance	Turbidity / $OD_{600\ nm}$
0	1.00 ± 0.14	1.00 ± 0.08	1.00 ± 0.05
30	0.84 ± 0.09	$(8.37 \pm 2.64)\times10^{-2}$	0.70 ± 0.12
60	0.91 ± 0.15	$(2.75 \pm 0.53)\times10^{-2}$	0.60 ± 0.09
90	1.37 ± 0.20	$(3.87 \pm 1.09)\times10^{-3}$	0.78 ± 0.21
120	2.16 ± 0.29	$(5.41 \pm 0.98)\times10^{-4}$	1.45 ± 0.31

Data are expressed as averages and standard deviations (n = 3).

RNA was extracted from INT and DEC spores at the different intervals during germination and outgrowth and its purity was determined spectrophotometrically from the ratio of absorption at 260 nm to that at 280 nm. All preparations were of high RNA purity, as indicated by a ratio in the range of 1.78 to 2.05, determined by the NanoDrop method, and of 1.73 to 2.04, determined by the GeneQuant method, resulting in a RIN number of 9.0 - 9.7. There was no difference in the purity of RNA from INT and DEC spores. The yield of extracted RNA depended on the status of the spores, whether they were dormant, germinating and outgrowing (Fig. 3-3). It is interesting to note that with the method applied it was possible to extract RNA of high purity even from dormant spores (at 0 min of incubation), although the yield was rather low (1 - 2 µg of RNA were isolated from 10^8 dormant spores). There was no statistically significant difference in the yield of RNA, whether extracted from INT or DEC dormant spores. With the initiation of germination of the spores the yield of extracted RNA increased. This

increase may be based on two processes: (i) an increasing susceptibility of the spore envelope to the lysing agents applied thereby easing the release of the RNA, and/or (ii) the initiation of RNA synthesis (Setlow, 2003). With increasing incubation time, the yield of RNA further increased. For INT spores it was doubled every 30 min of incubation (Fig. 3-3). As followed from the CFU and OD data (Fig. 3-2), at these periods of incubation, growth and cell replication had already started. For DEC spores, the increase in the amount of extracted RNA with incubation time was less pronounced, although the differences in both curves (INT and DEC in Fig. 3-3) are not statistically significant at most time points.

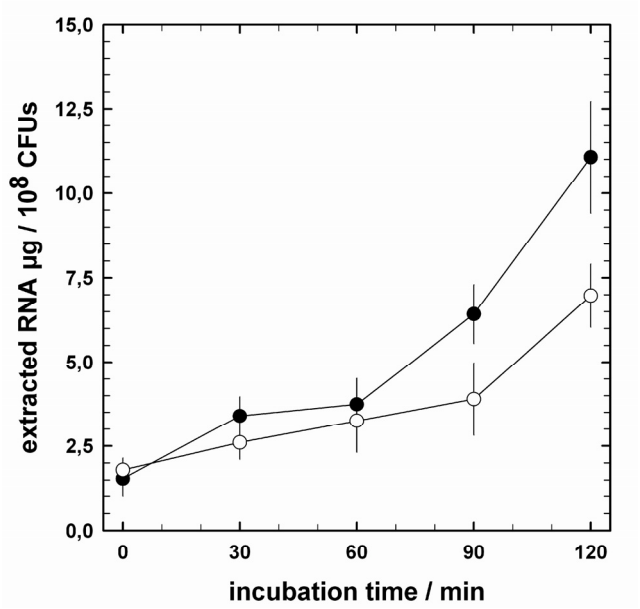


Fig. 3-3 Yield of RNA in µg/10^8 spores or cells extracted from spore coat intact (solid circles) or chemically decoated (open circles) spores that were dormant or in different stages of germination and outgrowth. Data are averages +/- standard deviation (n = 4).

The yields of extracted RNA of both spore types were almost identical for 0 to 60 min of incubation. Between 90 and 120 min the outgrowing INT spores showed approximately 1.5 - 2 times more extractable RNA than the outgrowing DEC spores. The final yield of RNA after 120 min of incubation was about 7 ± 1.5 µg/10^8 cells for DEC spores, whereas this values was about 11 ± 2.7 µg/10^8 cells for INT spores. For comparison, RNA isolation from vegetative *B. subtilis* cells performed by common commercial RNA extraction kits (i.e. Qiagen RNeasy Protect Bacteria Mini Kit, Duesseldorf, Germany) yielded 10 - 20 µg RNA/10^8 cells (Au et al., 2005; Goranov et al., 2006).

The RNA from both INT and DEC spores was separated in a 2100 BioAnalyzer (Fig. 3-4). In all cases, densitometric tracings of the gel images showed that 16S and 23S rRNA species were represented by sharp main peaks at 39 s and 42 s retention time, respectively. The 5S rRNA peak was visible at 25 s retention time. Densitometric traces of the images (Fig. 3-4) showed that rRNA peaks were sharp and non-degraded. In addition, several distinct minor peaks presumably representing individual mRNA species were also observed in the original gel-like images (not shown), demonstrating the high quality of the extracted RNA. Spectrophotometrical and densitometrical quantification evidenced that the isolated RNA is pure and free from cellular substances (i.e. DNA, proteins), that might disturb the subsequent molecular analysis.

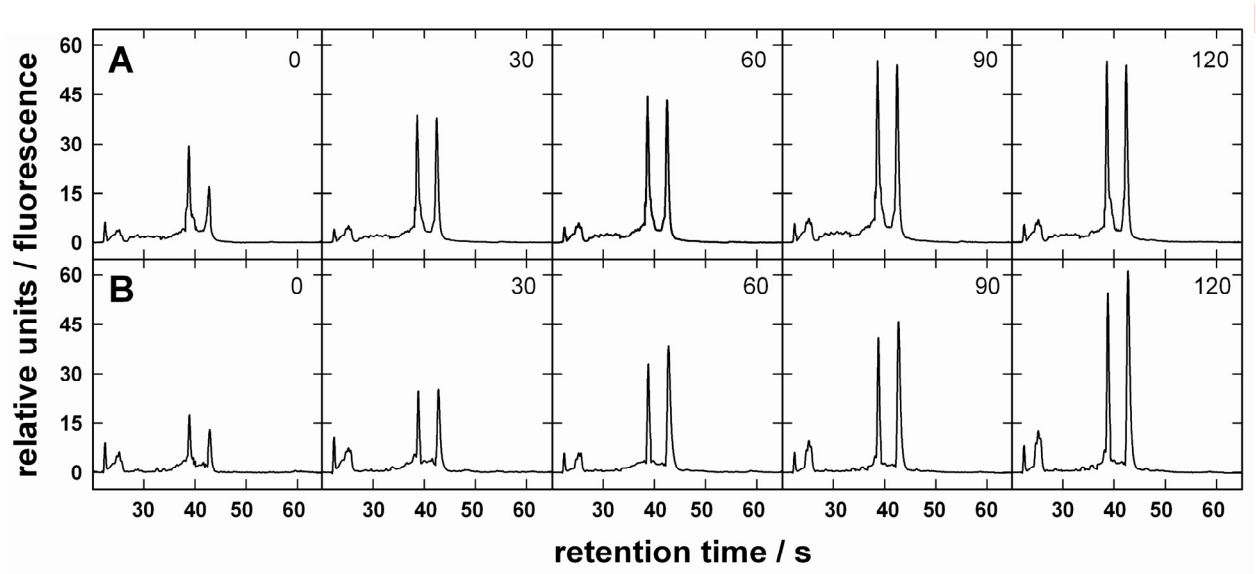


Fig. 3-4 Profiles of rRNA distribution determined in a BioAnalyzer from RNA preparations extracted from spore coat intact (**A**) and chemically decoated (**B**) *B. subtilis* spores that were dormant and spores that were in different stages of germination and outgrowth. The numbers in the right corner give the time during incubation when RNA was extracted.

These data show that the acid–phenol method used (2.1.8) is efficient to extract RNA from dormant bacterial endospores as well as from germinating and outgrowing spores. As judged from the yield of rRNA, 10^8 spores are sufficient for gaining the mRNA required for further studies, such as gene expression analyses. Although chemically decoated spores have been reported to have the advantage of easier access to DNA (Nicholson and Setlow, 1990), in these experiments the germination efficiency as

well as the efficiency of RNA extraction were impaired at all stages of germination of DEC compared to INT spores, although the differences were not statistically significant.

Therefore, in all further experiments, spore coat intact spores were taken for gene expression analysis experiments after DNA damaging treatments and spore germination. Only RNA samples with a RIN number above 9.0 were used for the gene expression studies.

3.2 Responses to UV-C radiation

Solar UV radiation has been considered as a major factor limiting spore survival in both, terrestrial surface and outer space environments (Horneck et al. 2001a; Setlow, 2001; Nicholson et al., 2005). Although the terrestrial as well as the extraterrestrial UV radiation is of polychromic nature, most studies of spore UV resistance have been restricted to investigating the effects of monochromatic 254-nm UV-C on spores of *B. subtilis* 168, and only few deal with other ranges of the UV spectrum e.g. the terrestrial UV ≥ 290 nm (Hullo et al., 2001).

3.2.1 Survival and induction of DNA bipyrimidine photoproducts upon UV-C irradiation

Spores of wild-type *B. atrophaeus* (DSM 675) and *B. subtilis* 168 strains, as well as *B. subtilis* strains carrying either deletions of the genes encoding α/β-SASP (*sspA sspB*), or deletions in genes encoding components of the major DNA repair pathways (spore photoproduct lyase, SP lyase: *splB*; nucleotide excision repair, NER: *uvrB*; recombination-mediated repair: *recA*), were exposed to 254-nm UV-C radiation as described in 2.2.3.1. Spore UV inactivation kinetics and photoproduct production were assessed in the same samples (Fig. 3-5). The UV-C survival curves (2.5, 2.5.1) of the spores were either exponential (the *sspA sspB* strain and all repair deficient strains except *recA*) or showed a slight shoulder, as indicated by n (= extrapolation number, i.e. the intercept with the ordinate of the extrapolated semi-log straight line) > 1 (*B. subtilis* wild-type and *recA* strain, and *B. atrophaeus*; Fig. 3-5A and Table 3-4). The F_{10} values of the wild-type spores and the *recA* mutant (Table 3-4) are in good agreement with published data (compiled as LD_{90} by Nicholson et al. (2000)), whereas the F_{10} values of the *sspA sspB*, *splB uvrB* and *recA splB uvrB* strains were substantially higher, by a factor of 4-10, than published values (Mason and Setlow, 1986; Nicholson et al., 2000). It is well established that the sporulation conditions influence the UV responses of spores (Gould, 1983; reviewed in

Nicholson et al., 2000; 2005). Therefore it is important to mention that in this work spores of all strains were produced by the same method (equal aspects were applied for all other treatments i.e. exposure to UV-A, high vacuum and ionizing radiation) and this therefore would allow comparison of their UV resistance, whereas the literature data were compiled from different studies. The data show that spore UV-C resistance decreased in the order (wild-type > *recA* > *sspA sspB* > *splB uvrB* > *recA splB uvrB*), which is in agreement with previously-published data (Nicholson et al., 2000). Examination of the RF values (Table 3-4) shows that among the repair mechanisms, the *recA* system was the least important mechanisms for repairing UV-C damage induced in spores, whereas the combined bipyrimidine photoproducts repair mechanisms *splB* and *uvrB* had a higher repair efficiency, which was further ameliorated by the presence of the *recA* system.

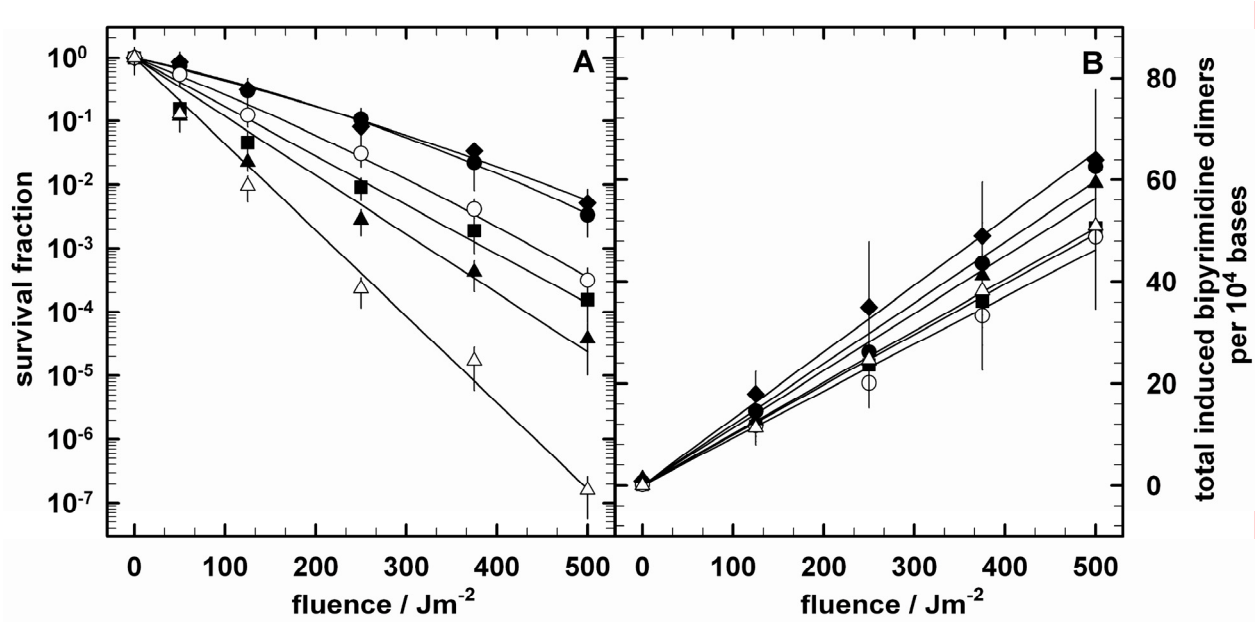


Fig. 3-5 Fluence-survival curve (**A**) and induction of bipyrimidine photoproducts (**B**) of *B. atrophaeus* DSM 675 (solid diamonds), *B. subtilis* 168 wild-type (solid circles), *sspA sspB* (solid squares), *recA* (open circles), *splB uvrB* (solid triangles) and *recA splB uvrB* (open triangles) after exposure to 254 nm UV-C radiation. Data are averages +/- standard deviation (n = 4).

The fluence dependent formation of bipyrimidine photoproducts (PP) induced in the DNA of UV-C irradiated spores is shown in Fig. 3-5B. The total amount of photoproducts/10^4 bases increased linearly with the applied fluence. It is interesting to note that the induction of the PP was slightly lower for the *recA*, *splB uvrB*, *recA splB uvrB*, and *sspA sspB* mutants than for the wild-type strains (Table 3-5). A

similar effect was observed by Douki et al. (2005a) for the latter strain (*sspA sspB*). However, the differences in k_{PP} were not statistically significant; thus, none of the mutations tested was found to significantly alter the overall quantum yield of total DNA photoproduct formation with respect to the wild-type strains.

Table 3-4 Survival curve characteristics after UV-C irradiation (254 nm)

UV-C (254 nm)	F_{10} (J/m²)	F_0 (J/m²)	F_q (J/m²)	IC (m²/J)
B. atrophaeus	238.1 ± 22.3	112.4 ± 18.2	17.4 ± 3.4	$(1.04 \pm 0.15)\times10^{-2}$
B. subtilis (wild-type)	225.3 ± 31.4	108.8 ± 17.3	20.3 ± 7.2	$(1.12 \pm 0.08)\times10^{-2}$
sspA sspB	104.2 ± 19.8	22.5 ± 5.2	n.d.	$(1.60 \pm 0.24)\times10^{-2}$
recA	153.3 ± 18.2	70.6 ± 10.1	7.3 ± 1.5	$(1.53 \pm 0.09)\times10^{-2}$
splB uvrB	77.3 ± 16.7	17.9 ± 4.3	n.d.	$(1.91 \pm 0.22)\times10^{-2}$
recA splB uvrB	61.0 ± 21.3	8.5 ± 2.4	n.d.	$(2.99 \pm 0.41)\times10^{-2}$

UV-C (254 nm)	n	RF	R^2	*P*
B. atrophaeus	1.56 ± 0.21	1.06 ± 0.09	0.987	0.243
B. subtilis (wild-type)	1.67 ± 0.26	1	0.989	
sspA sspB	0.87 ± 0.16	0.46 ± 0.07	0.981	0.041
recA	1.12 ± 0.23	0.68 ± 0.08	0.991	0.037
splB uvrB	0.73 ± 0.17	0.34 ± 0.12	0.986	0.029
recA splB uvrB	0.62 ± 0.20	0.27 ± 0.05	0.993	0.015

n.d. not determinable. The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). *P* = statistical significance of difference of data of respective strain compared to *B. subtilis* wild-type strain 168; $P \leq 0.05$ = significant difference. Data are averages and standard deviations ($n = 5$).

Using HPLC-MS/MS, it was possible to determine not only the total amount of bipyrimidine photoproducts, but also to identify and quantify each individual type of photoproduct formed in the UV-irradiated spores (2.3.1). The results are compiled in Table 3-5. SP was the predominant DNA photoproduct (93 ± 5 %) in all spores with wild-type SASP formation. CPD TC, CPD TT and CPD CT

as well as 6-4 TC and 6-4 TT were also detected; however they were produced in small fractions (in a range of 5 - 7 % of the total bipyrimidine photoproducts).

Table 3-5 Yields of photolesions in *Bacillus* sp. spores exposed to UV-C radiation

Strain	SP (TDHT)	CPD TC	CPD TT	6-4 TC
B. atrophaeus	$(1.4 \pm 0.4)\times10^{-1}$	$(3.9 \pm 0.3)\times10^{-3}$	$(2.8 \pm 0.7)\times10^{-3}$	$(2.9 \pm 0.4)\times10^{-3}$
B. subtilis (wild-type)	$(1.1 \pm 0.2)\times10^{-1}$	$(3.2 \pm 0.2)\times10^{-3}$	$(3.1 \pm 0.6)\times10^{-3}$	$(2.5 \pm 0.3)\times10^{-3}$
sspA sspB	$(0.5 \pm 0.1)\times10^{-1}$	$(1.2 \pm 0.1)\times10^{-2}$	$(2.3 \pm 0.2)\times10^{-2}$	$(1.3 \pm 0.3)\times10^{-2}$
recA	$(0.9 \pm 0.3)\times10^{-1}$	$(4.2 \pm 0.2)\times10^{-3}$	$(2.4 \pm 0.5)\times10^{-3}$	$(3.3 \pm 0.4)\times10^{-3}$
splB uvrB	$(1.0 \pm 0.2)\times10^{-1}$	$(2.2 \pm 0.3)\times10^{-3}$	$(2.8 \pm 0.7)\times10^{-3}$	$(2.6 \pm 0.5)\times10^{-3}$
recA splB uvrB	$(0.9 \pm 0.2)\times10^{-1}$	$(3.1 \pm 0.3)\times10^{-3}$	$(4.0 \pm 1.1)\times10^{-3}$	$(2.8 \pm 0.4)\times10^{-3}$

Strain	CPD CT	6-4 TT	Total	total PP / 1 chromosome[a]
B. atrophaeus	$(8.6 \pm 2.1)\times10^{-4}$	$(5.2 \pm 1.7)\times10^{-4}$	$(1.4 \pm 0.4)\times10^{-1}$	$(5.9 \pm 1.2)\times10^{4}$
B. subtilis (wild-type)	$(7.2 \pm 1.7)\times10^{-4}$	$(4.8 \pm 1.4)\times10^{-4}$	$(1.2 \pm 0.2)\times10^{-1}$	$(5.1 \pm 0.8)\times10^{4}$
sspA sspB	$(1.5 \pm 0.1)\times10^{-3}$	$(1.8 \pm 0.4)\times10^{-3}$	$(0.9 \pm 0.3)\times10^{-1}$	$(3.8 \pm 1.2)\times10^{4}$
recA	$(8.3 \pm 2.3)\times10^{-4}$	$(5.5 \pm 1.9)\times10^{-4}$	$(1.0 \pm 0.3)\times10^{-1}$	$(4.2 \pm 0.7)\times10^{4}$
splB uvrB	$(6.9 \pm 1.8)\times10^{-4}$	$(2.9 \pm 0.7)\times10^{-4}$	$(1.1 \pm 0.2)\times10^{-1}$	$(4.6 \pm 0.6)\times10^{4}$
recA splB uvrB	$(6.7 \pm 3.1)\times10^{-4}$	$(7.0 \pm 2.5)\times10^{-4}$	$(1.0 \pm 0.3)\times10^{-1}$	$(4.2 \pm 0.9)\times10^{4}$

Data are expressed as k_{PP} = lesions/10^4 bases per Jm^{-2}. [a]DNA PP level calculated on the chromosome size of *B. subtilis* 168 after 0.5 kJ/m² 254 nm UV-C (according to the genomic information published by Kunst et al., 1997). Data are averages and standard deviations (n = 4).

After 500 J/m² 254 nm UV-C radiation roughly 47 000 DNA bipyrimidine photoproducts were determined in every single spore chromosome independently of the spore genotype. Whereas the amount of total DNA photoproducts was similar in spores of the *sspA sspB* strain as in those possessing small acid-soluble proteins (SASPs), the spectrum of induced photoproducts was significantly altered compared to the pattern observed in the wild-type. In *sspA sspB* spores, SP was still the main DNA lesion with 48 ± 6 % of the overall photoproducts, and the residual photoproducts were distributed among CPDs (CPD TT 24 ± 5 %, CPD TC 13 ± 8 %, CPD CT 2 ± 1 %) and 6-4 PPs

(6-4 TT 2 ± 1 %, 6-4 TC 13 ± 7 %). In Fig. 3-6 the bipyrimidine photoproduct distribution of UV-C irradiated wild-type SASPs and SASPs deficient spores are shown.

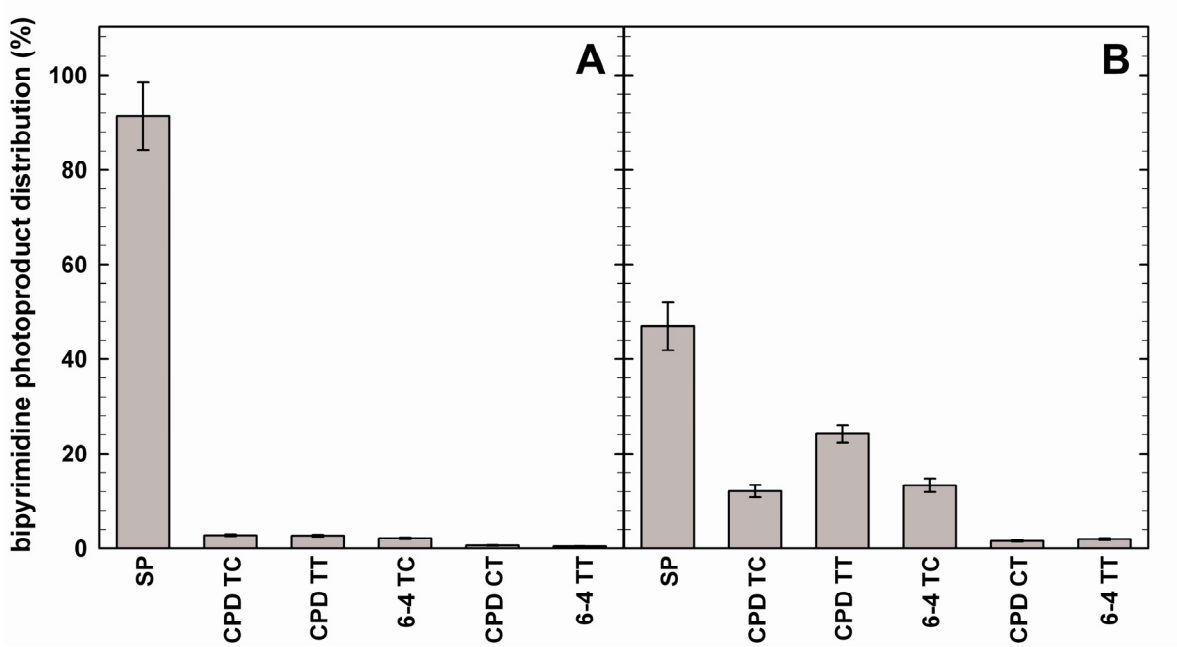


Fig. 3-6 Distribution of the induced bipyrimidine photoproducts following UV-C irradiation of small, acid-soluble spore proteins (SASP) wild-type (**A**) (*B. subtilis* 168) and major SASPs deficient (**B**) (*sspA sspB*) spores. Data are expressed as percentage of total photoproducts, and are reported as averages and standard deviations (n = 4).

3.2.2 Repair of DNA (UV-C) photolesions during spore germination

In order to monitor the ability of wild-type and mutant spores to repair UV-C induced DNA damage during germination, spores of each strain were irradiated with 254 nm UV-C at a fluence of 125 J/m^2. Then the spores were germinated in 2 x LB medium with *L*-alanine, and the kinetics of photoproduct repair during germination was assessed (2.1.6). To monitor germination, samples were taken in parallel for the determination of optical density and germination-associated loss of heat resistance. Each strain showed normal spore germination kinetics as monitored by typical optical density measurements, loss of heat resistance and low cell replication activity during the first two hours of germination (Tables 3-1 and 3-2). In short, spores of all strains germinated at approximately the same rate and extent, in good agreement with published data (Setlow and Setlow, 1996).

The repair kinetics of the total bipyrimidine photoproducts, of spore photoproduct (SP) and of CPD TT showed a first-order exponential decrease with germination time (Fig. 3-7). *B. atrophaeus*, *B. subtilis* 168 and the *sspA sspB* spores showed nearly equally efficient rates of overall repair of all bipyrimidine dimers, whereas little or nearly no repair of the total photoproducts was achieved during germination of *splB uvrB* and *recA splB uvrB* mutant spores. After 1 h of germination, a high percentage of total induced photolesions was repaired in *B. atrophaeus* (72 ± 15 %) and *B. subtilis* 168 (73 ± 10 %), and in the α/β-type SASP deficient strain (68 ± 18 %) (Fig. 3-7).

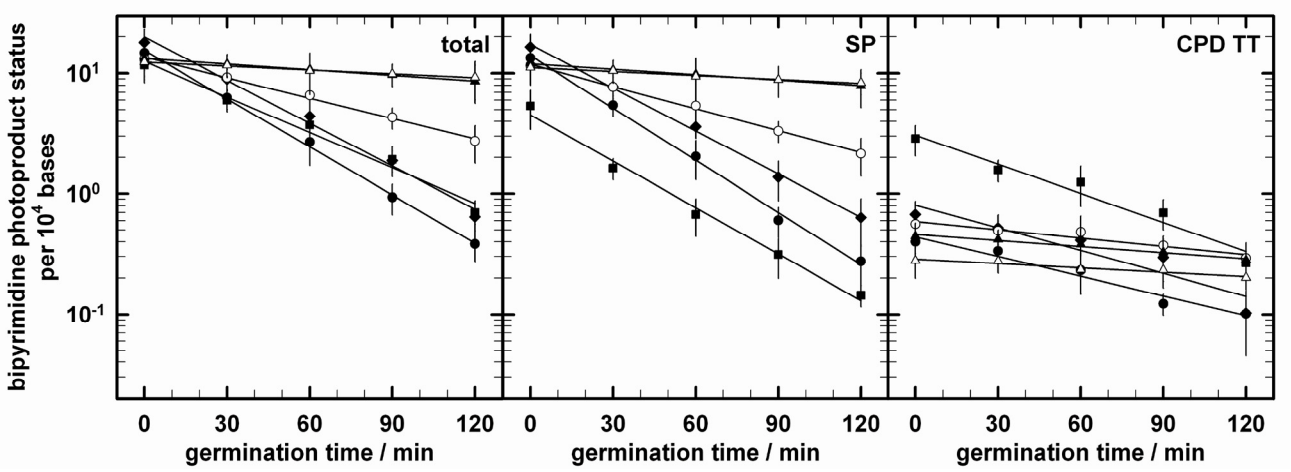


Fig. 3-7 Repair kinetics of total bipyrimidine photoproducts, the spore photoproduct SP, and CPD TT during germination of *B. atrophaeus* (solid diamonds), *B. subtilis* wild-type (solid circles), *sspA sspB* (solid squares), *recA* (open circles), *splB uvrB* (solid triangles) and *recA splB uvrB* (open triangles) spores exposed to 125 J/m^2 of UV-C radiation. Data are reported as averages and standard deviations (n = 6).

The removal of total photoproducts from the DNA of the *recA* strain was with 49 ± 15 % significantly lower during the same period of germination (Table 3-6). Negligible, but still measurable, photoproduct removal was achieved in germinating *splB uvrB* spores (19 ± 8 %) or *recA splB uvrB spores* (15 ± 9 %) after 1 h of germination. It should be noted that these repair studies were performed with spores exposed to a relatively low UV fluence (125 J/m²) which was well below the F_{10} values of the wild-type strains.

Table 3-6 Repair of bipyrimidine PP in spore DNA of wild-type and mutant *Bacillus* sp. strains

Strain	SP (TDHT)	CPD TC	CPD TT	6-4 TC
B. atrophaeus	78.1 ± 12.1	69.1 ± 13.0	39.1 ± 7.5	40.4 ± 7.7
B. subtilis (wild-type)	84.7 ± 13.8	76.0 ± 7.1	41.9 ± 12.2	26.4 ± 9.1
sspA sspB	83.1 ± 11.4	55.8 ± 18.3	56.8 ± 9.5	47.7 ± 10.6
recA	53.1 ± 7.6	34.2 ± 9.5	14.2 ± 7.1	28.5 ± 9.3
splB uvrB	18.8 ± 10.5	29.2 ± 12.3	14.9 ± 5.6	25.2 ± 14.2
recA splB uvrB	15.3 ± 8.3	8.2 ± 5.3	15.5 ± 10.1	13.1 ± 8.3

Strain	CPD CT	6-4 TT	Total
B. atrophaeus	43.1 ± 15.6	46.9 ± 8.7	71.5 ± 14.6
B. subtilis (wild-type)	62.4 ± 20.1	83.4 ± 16.6	73.2 ± 10.4
sspA sspB	39.2 ± 8.7	47.8 ± 9.8	68.1 ± 18.2
recA	28.2 ± 10.3	45.6 ± 14.9	49.4 ± 15.2
splB uvrB	23.7 ± 12.7	27.6 ± 10.6	19.2 ± 13.1
recA splB uvrB	19.8 ± 9.5	19.2 ± 9.8	15.1 ± 9.6

Measurements were made at 60 minutes of germination and expressed as percent of photoproduct repaired relative to time zero of germination. Data are averages and standard deviations (n = 6).

For the direct comparison of the DNA repair capability of all tested genotypes, the DNA bipyrimidine photoproduct repair index, the reciprocal value of the slope in Fig. 3-7, was determined (Fig. 3-8). Both wild-type spores, *B. subtilis* and *B. atrophaeus*, and the *sspA sspB* strain show the highest DNA PP repair efficiency, i.e. 367 ± 41 total PP/min (per single chromosome) in a time-frame of 120 germination and outgrowth. While the *recA*, *splB uvrB* and *recA splB uvrB* were only capable to repair 267 ± 34, 74 ± 17 and 45 ± 10 of the total induced PP/min, respectively in the same period. Similar tendencies were obtained by analyzing the genotype specific repair constant, as can see in Table 3-7.

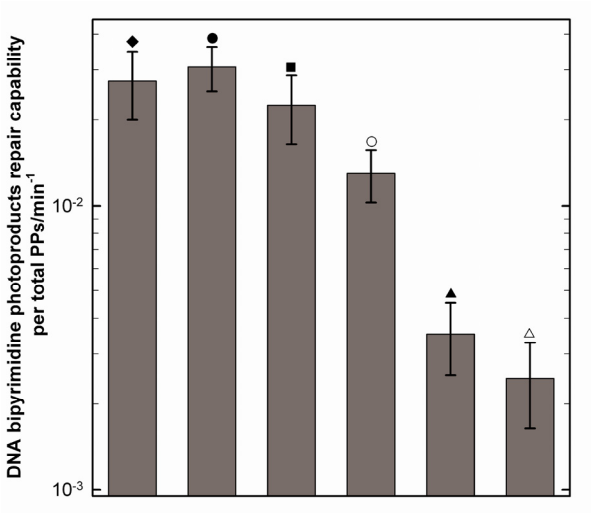


Fig. 3-8 DNA bipyrimidine photoproduct repair efficiency of germinating and outgrowing UV-C irradiated (125 J/m^2) endospores *B. atrophaeus* (solid diamonds), *B. subtilis* wild-type (solid circles), *sspA sspB* (solid squares), *recA* (open circles), *splB uvrB* (solid triangles) and *recA splB uvrB* (open triangles). Data are expressed as averages from the total repaired photoproduct quantity per recovery time (PP/min) and standard deviations (n = 6).

Table 3-7 SP repair efficiency during spore germination of wild-type and mutant *Bacillus* sp. strains

Strain	RC	$RC_{wild\text{-}type}$ / $RC_{rep.\ def.\ strains}$
B. atrophaeus	$(1.19 \pm 0.21)\times10^{-2}$	0.91 ± 0.11
B. subtilis (wild-type)	$(1.32 \pm 0.30)\times10^{-2}$	1
sspA sspB	$(1.28 \pm 0.17)\times10^{-2}$	0.96 ± 0.08
recA	$(6.09 \pm 1.47)\times10^{-3}$	0.46 ± 0.09
splB uvrB	$(1.12 \pm 0.23)\times10^{-3}$	0.08 ± 0.03
recA splB uvrB	$(0.83 \pm 0.24)\times10^{-3}$	0.06 ± 0.02

Repair effiency was obtained from the slope of the SP repair curve in Fig. 5-7. Data are averages and standard deviations (n = 6).

3.2.3 Transcriptional profile of UV-C irradiated germinating *B. subtilis* spores

In order to understand the repair mechanisms of DNA damage induced by UV-C in dormant spores a genome-wide gene expression analysis was performed during germination of *B. subtilis* spores. For the analysis of the transcriptional response during spore germination, samples for RNA isolation were rapidly drawn and snap frozen. The method used for the RNA isolation and quantification from dormant, germinating and outgrowing spores is described in detail in 2.1.8 (Moeller et al., 2006; 2007b; Keijser et al., 2007). RNA isolated from germinating spores was used to prepare fluorescently labeled cDNA which was hybridized onto 65-mer oligonucleotide slides. After washing and scanning, fluorescence intensity data were extracted and analyzed. The global transcriptional profile of

germinating *B. subtilis* 168 spores under radiation stress conditions was obtained using DNA microarray-based measurements. To monitor changes in gene expression the mRNA profiles of 60 min (and 30 min only for wild-type spores, respectively) germinating wild-type, *recA* (recombination-mediated repair) and *splB uvrB* (spore photoproduct lyase, SP lyase and nucleotide excision repair, NER) spores after UV-C radiation (125 J/m^2) stress were imposed with the profiles of no-irradiated spores from the respective strain. The majority of comparisons were performed in at least four independent experiments. Microarray data processing (fluorescent intensity differences of the ratio UV-C irradiated *versus* non-irradiated germinating spores), statistical significance data analysis and functional interpretation of the microarray data were performed as described in detail in chapter 2.4 (2.4.4 - 2.4.6).

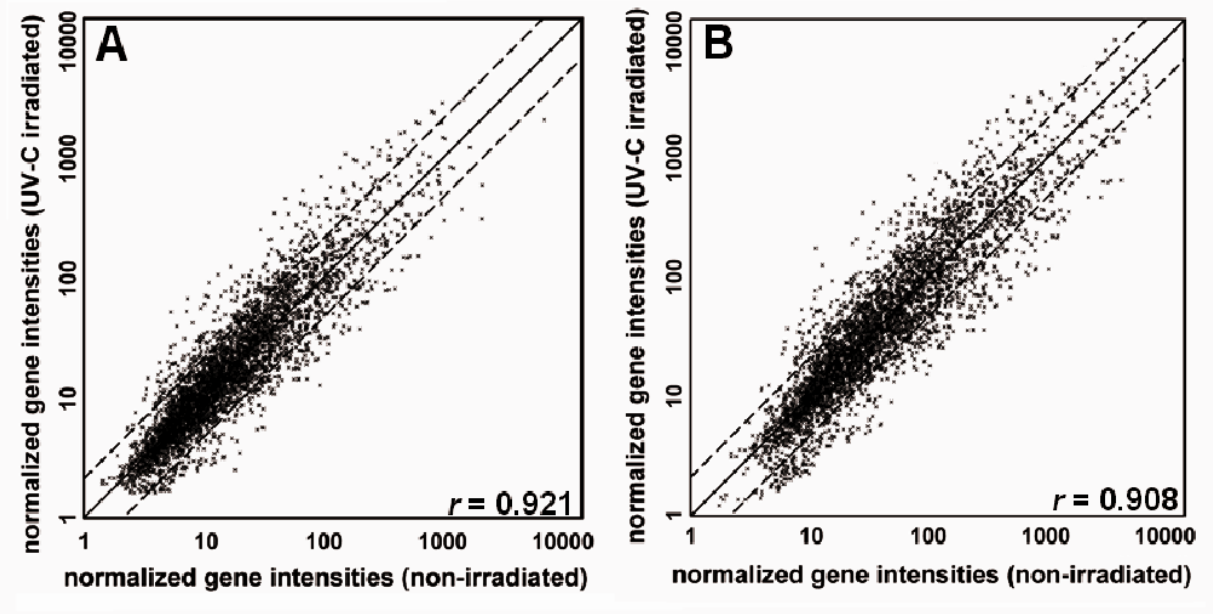


Fig. 3-9 Scatter diagrams of normalized spot intensities. Gene intensities are plotted for 254-nm UV-C irradiated and non-irradiated wild-type (**A**) and *recA*-deficient (**B**) spores after 60 min germination. The significance threshold of twofold induction or repression is symbolized by the two dashed lines. Data are expressed as mean values (n = 4). r = Pearson correlation coefficient.

Prior to quantification of the array data, the quality and reproducibility of the array experiment were estimated by comparing the normalized spot intensities in the scattered diagram for 60 min germinating wild-type and *recA*-deficient spores (Fig. 3-9, as selected example for UV-C irradiated, germinating spores). Array data from hybridization of independent samples representing the same cultivation

condition always yielded high Pearson correlation coefficient with $r \geq 0.900$ (Blencke et al., 2003; Moeller et al., 2007b), measured for three tested strains (wild-type (0.921), *recA* (0.908) and *uvrB*/*splB* (0.903; scatter diagram not shown)).

Using a 2-fold balanced differential expression as the appropriate threshold level, 432 of the 4107 known and unknown ("*y*"-) genes were found to be up-regulated in the wild-type strain (10.5 %), whereas only 263 for the *recA*-deficient (6.4 %) and 241 for the *uvrB*/*splB*-deficient spores (5.8 %) genes were induced after UV-C irradiation during spore germination. Table 3-8 lists the 144 up-regulated genes (with a significant ≥ 2.5-fold change), determined from the 60 min-germinated wild-type spore population and correspondingly presented for 30 min-germinated wild-type spores and 60 min-germinated *recA* and *uvrB*/*splB* spore population. In addition for all these genes the putative LexA-binding site according to Au et al. (2005) and their response to further DNA damaging agents, e.g. mitomycin C were determined by bibliographic search (for detailed information see footnotes in Table 3-8).

Table 3-8 UV-C radiation-induced genes after 30[1] and 60[2] min germination

Gene[a]	Function[b]	Fold induction[c]			LexA-bind. site[d]	Induced by further stimulons?[e]
		[1/2]w.t.	[2]*recA*	[2]*uvrB*/*splB*		
dnaA	initiation of chromosome replication	3.1 / 4.7	2.4	1.9		MMC, UV
recF	DNA repair and genetic recombination	1.9 / 4.0	1.4	2.4	+	MMC, UV
gyrB	DNA gyrase (subunit B)	2.4 / 3.6	1.9	2.1		
dnaX	DNA polymerase III (gamma and tau subunits)	1.8 / 4.2	2.4	3.0		
purR	transcriptional repressor of the purine operons	1.4 / 3.0	2.0	2.2		
yabR	unknown; similar to polynucleotide phosphorylase / polyadenylase [*Escherichia coli* K12, E value 8e-16]	1.5 / 3.8	1.4	1.8		
yazB	unknown; similar to DNA-binding protein [*B. anthracis* str. Ames, E value 5e-20]	2.5 / 4.7	1.8	1.4		
yacK	unknown; similar to DNA-binding protein / DNA integrity scanning protein (DisA) [*B. licheniformis* ATCC 14580, E value 1e-172]	1.6 / 3.3	2.2	1.7		GS, HS
adk	adenylate kinase	1.5 / 2.7	1.4	2.0		
infA	initiation factor IF-1	2.0 / 2.6	1.3	1.4		
sigW	RNA polymerase ECF-type sigma factor	1.6 / 3.8	2.2	1.9		
ndhF	NADH dehydrogenase (subunit 5)	1.9 / 4.1	2.4	1.9		
dinB	nuclease inhibitor	5.1 / 11.4	1.9	3.1	+	MMC, PS, UV
pbuG	hypoxanthine / guanine permease	1.3 / 5.5	1.8	2.4		
purK	phosphoribosylaminoimidazole carboxylase II	1.1 / 2.5	1.9	2.0		
yerB	unknown; similar to DNA mismatch repair protein [*Plasmodium yoelii yoelii* str. 17XNL, E value 9.1]	1.5 / 2.8	1.4	1.5		

yerH	unknown; similar to DNA mismatch repair protein [*Geobacillus kaustophilus* HTA426, E values 1.4]; spore cortex-lytic enzyme prepeptide [*B. cereus* G9241, E value 4.1]	1.8 / 5.1	1.8	2.2	+	
prsA	protein secretion (post-translocation molecular chaperone)	1.5 / 3.0	1.5	2.0		
yjcD	unknown; similar to ATP-dependent DNA helicase, UvrD/REP family [*B. clausii* KSM-K16, E value 1e-169]	4.4 / 9.7	1.4	1.2		
yjcR	unknown; similar to DNA binding protein [*B. licheniformis* ATCC 14580, E values 5e-52]; resolvase-like helix-turn-helix domain protein [*Solibacter usitatus* Ellin6076, E value 0.077]	2.0 / 6.3	1.7	2.4		
yjlC	unknown; similar to adenine specific DNA methyltransferase [*Nodularia spumigena* CCY9414, E value 5.9]	1.9 / 3.4	1.8	2.0		AG
ykoQ	unknown; similar to DNA repair exonuclease / phosphoesterase [*B. cereus* E33L, E value 6e-91]	2.5 / 7.3	3.3	2.4		
clpE	ATP-dependent Clp protease-like (class III stress gene)	1.2 / 4.0	2.6	3.0		GS, HS
ykvM	unknown; similar to GTP cyclo-hydrolase I [*B. licheniformis* ATCC 14580, E value 1e-93]	1.6 / 3.5	1.2	1.8		
ykvT	unknown; similar to spore cortex-lytic enzyme [*Geobacillus kaustophilus* HTA426, E value 4e-28]	1.8 / 3.0	1.5	2.4		PS
ykuK	unknown; similar to exonuclease subunit C [*C. difficile* 630, E value 5e-73]	2.7 / 7.4	1.8	2.0		
ylaG	unknown; similar to GTP-binding protein TypA (stress response GTPase) [*Lactobacillus sakei* subsp. *sakei* 23K, E value 0.0]	2.1 / 2.5	2.1	1.9		
ylaH	unknown; similar to uracil-xanthine permease [*Chromohalobacter salexigens* DSM 3043, E value 8.9]	1.4 / 3.4	1.8	2.5		
ylbH	unknown; similar to N^6-adenine-specific DNA methylase [*B. licheniformis* ATCC 14580, E value 8e-64]	1.8 / 6.7	1.9	4.2		
murG	UDP-N-acetylglucosamine-N-acetyl-muramyl-(pentapeptide)-pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	1.4 / 4.5	2.0	3.0		GS
yloC	unknown; similar to ATP-dependent DNA helicase (RecG) [*Saccharophagus degradans* 2-40, E value 1e-31]; uncharacterized stress-induced protein [*B. anthracis* str. A2012, E value 1e-61]	2.3 / 6.4	1.6	2.5		
ylzA	unknown; similar to oxidoreductase protein; putative dehydrogenase (flavoprotein) [*Acinetobacter* sp. ADP1, E value 7.2]	1.5 / 3.4	1.4	1.9		
gmk	guanylate kinase	1.3 / 2.8	1.5	1.8		
yloV	unknown; similar to predicted kinase related to hydroxyacetone kinase [*B. cereus* ATCC 14579, E value 0.0]; putative phosphatase [*Staphylococcus aureus* subsp. *aureus* Mu50, E value 1e-171]	1.8 / 3.2	1.4	2.0		
ylxM	unknown; similar to putative helix-turn-helix protein, YlxM / p13-like [*B. cereus* subsp. *cytotoxis* NVH 391-98, E value 1e-24]; DNA-binding protein [*Streptococcus mutans* UA159, E value 3e-18]	2.5 / 4.9	1.8	2.2		
clpY	two-component ATP-dependent protease [ClpQ]	1.5 / 2.7	1.4	1.8		GS, HS
pyrH	uridylate kinase	1.8 / 4.1	1.8	1.6		
frr	ribosome recycling factor	1.2 / 2.6	1.5	2.0		SA

recA	multifunctional protein involved in homologous recombination and DNA repair (LexA-autocleavage)	8.1 / 23.2	1.7	6.3	+	MMC, PS, UV
ymdA	unknown; similar to metal dependent phosphohydrolase [*Desulfitobacterium hafniense* DCB-2, E value 1e-161]	1.4 / 5.0	1.9	2.0		
ymcA	unknown; similar to chromosome segregation protein SMC [*C. novyi* NT, E value 0.003]; UvrB/UvrC protein:AAA ATPase, central region: Clp, N terminal [*Exiguobacterium sibiricum* 255-15, E value 0.18]	2.1 / 4.0	1.4	2.0		
ymaB	unknown; similar to phosphoesterase [*B. clausii* KSM-K16, E value 7e-60]; MutT/nudix family protein [*Staphylococcus epidermidis* RP62A, E value 9e-34]	1.7 / 4.6	2.6	1.4		MMC
yncD	unknown; similar to alanine racemase region [*B. cereus* subsp. *cytotoxis* NVH 391-98, E value 2e-86]	1.4 / 3.4	1.9	2.8		
lexA	transcriptional repressor of the SOS regulon	3.8 / 5.3	1.3	3.4	+	MMC, PS, UV
yneB	unknown; similar to resolvase, N-terminal domain – putative recombinase [*B. licheniformis* ATCC 14580, E value 4e-90]; site-specific recombinase [*B. cereus* ATCC 14579, E value 9e-78]	5.5 / 10.4	1.7	2.3	+	MMC, PS, UV
yoeB	unknown; similar to DNA-3-methyladenine glycosylase I [*Dictyostelium discoideum* AX4, E value 1.2]; DNA topoisomerase III [*Listeria monocytogenes* str. 4b H7858, E value 3.6]	2.1 / 7.3	1.8	2.4		GS, PS, SA^R
yobH	unknown; similar to UV-damage repair protein (UvrX) [UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-108]; ImpB/MucB/SamB family protein [Bacteriophage SPBc2, E value 1e-108]	4.1 / 8.9	1.2	1.6	+	MMC, UV
yozK	unknown; similar to UV-damage repair protein (uvr) [*B. subtilis subsp. subtilis* str. 168]; ImpB/MucB/SamB family protein [Bacteriophage SPBc2, E value 7e-58]	4.1 / 13.0	1.5	2.2	+	
yozL	unknown; similar to YolD homolog [*B.* sp. NRRL B-14911, E value 0.009]	3.2 / 8.0	1.8	2.0	+	MMC, UV
yobL	unknown; similar to molecular chaperone DnaK [*Helicobacter hepaticus* ATCC 51449, E value 0.034]	1.2 / 2.9	1.4	1.7		
yocG	unknown; similar to DNA-binding response regulator [*B. anthracis* str. Ames, E value 5e-68]	1.8 / 7.8	3.2	2.4		
yocH	unknown; similar to cell wall-binding protein [*Oceanobacillus iheyensis* HTE831, E value 5e-46]; *N*-acetylmuramoyl-*L*-alanine amidase [*Oceanobacillus iheyensis* HTE831, E value 3e-11]	1.4 / 5.2	3.2	4.1		SA
yocI	unknown; similar to ATP-dependent DNA helicase (RecQ) [*B. cereus* ATCC 14579, E value 0.0]	2.1 / 6.2	1.5	2.3		
yocJ	unknown; similar to acyl carrier protein phosphodiesterase [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-115]	1.4 / 3.7	1.8	2.4		
yotL	unknown; similar to putative DNA-binding protein [*Aeromonas hydrophila* subsp. *hydrophila* ATCC, E value 0.027]	1.6 / 3.5	1.2	2.4		
yosH	unknown; similar to DNA polymerase III, alpha subunit, gram-positive type [*C. perfringens* ATCC 13124, E value 0.84]	1.7 / 2.5	2.4	2.3		
yorS	unknown; similar to 5'(3')-deoxyribonucleotidase [*Robiginitalea biformata* HTCC2501, E value 4e-29]; DNA polymerase alpha [*Plasmodium falciparum*, E value 1.8]	1.1 / 2.8	2.0	1.8		

yorR	unknown; similar to deoxypurine (deoxyguanosine / deoxyadenosine) kinase subunit [*Oceanobacillus iheyensis* HTE831, E value 3e-04]	1.6 / 4.1	1.3	1.7	
yorL	unknown; similar to DNA polymerase III, alpha subunit, truncation [*Staphylococcus epidermidis* RP62A, E value 1e-141]	1.4 / 3.0	1.4	2.1	+
yorK	unknown; similar to single-stranded-DNA-specific exonuclease (RecJ) [*Staphylococcus epidermidis* RP62A, E value 1e-54]	1.7 / 5.6	1.9	2.3	
yorJ	unknown; similar to DNA primase [*C. botulinum* phage C-St, E value 9e-18]	1.5 / 2.6	2.4	2.0	
yorH	unknown; similar to excinuclease ABC subunit A [*B. halodurans* C-125, E value 0.034]	1.4 / 6.8	1.4	1.1	
yorG	unknown; similar to ATP/GTP binding protein [Bacteriophage SPBc2, E value 0.0]	1.2 / 3.8	1.8	3.0	+
yorF	unknown; similar to conserved hypothetical protein [*Staphylococcus epidermidis* RP62A, E value 5e-15]	1.4 / 2.5	1.1	2.6	+
yorC	unknown; similar to DNA polymerase III gamma and tau subunits (dnaX) [*Campylobacter lari* RM2100, E value 6.2]	1.7 / 4.1	1.6	2.0	+
yoqJ	unknown; similar to DNA mismatch repair protein MutS [*Pseudomonas aeruginosa* UCBPP-PA14, E value 6.9]	1.3. / 5.7	2.6	3.7	
yopJ	unknown; similar to ATP-dependent DNA helicase PcrA, putative [*C. perfringens* SM101, E value 0.14]	1.5 / 4.9	2.0	1.3	
yonX	unknown; similar to DNA recombination protein RmuC [*Gramella forsetii* KT0803, E value 0.31]	1.8 / 4.5	1.4	2.4	
yomV	unknown; similar to putative DNA helicase [*Prochlorococcus marinus* str. MIT 9515, E value 6.6]	1.4 / 6.7	1.6	1.9	
yomU	unknown; similar to DNA-dependent ATPase SNF2H [*Mus musculus*, E value 0.98]	1.4 / 2.6	1.8	2.4	
yomO	unknown; similar to ATP-binding region, ATPase-like: histidine kinase A, N-terminal [*C. beijerincki* NCIMB 8052, E value 2.1]	1.3 / 2.8	1.5	1.8	
yomM	unknown; similar to site-specific recombinase, phage integrase family [*Staphylococcus epidermidis* RP62A, E value 4e-97]	1.4 / 2.5	1.8	1.5	
yomH	unknown; similar to DNA polymerase III subunits gamma and tau [*Helicobacter acinonychis* str. Sheeba, E value 4.4]	1.8 / 3.5	2.4	3.1	
yomG	unknown; similar to DNA double-strand break repair rad50 ATPase [*Methanococcus maripaludis* S2, 2e-04]; DNA repair protein (RecN) [*Streptococcus agalactiae* 2603V/R, E value 3e-04]	1.7 / 5.4	1.6	2.4	
volF	unknown; similar to phenylalanine tRNA synthetase like beta subunit [*Strongylocentrotus purpuratus*, E value 3.0]	1.4. / 2.8	1.6	2.0	+
uvrX	ImpB/MucB/SamB family protein [Bacteriophage SPBc2]; UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168 E value 0.0]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 1e-103]	5.8 / 18.7	1.4	3.4	+
volD	unknown; similar to putative chromosome segregation protein, SMC ATPase superfamily [*Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986, E value 5.1]; cyclic nucleotide-binding domain containing protein [*Tetrahymena thermophila* SB210, E value 8.7]	1.5 / 2.8	1.8	2.3	+

yolB	unknown; similar to endonuclease [*Campylobacter jejuni* subsp. *jejuni* NCTC 11168, E value 6.5]	1.3 / 2.6	2.5	1.4		
yolA	unknown; similar to helicase (RecQ) [*Pasteurella multocida* subsp. *multocida* str. Pm70, E value 5.5]	1.5 / 4.0	1.6	3.2		
yokH	unknown; similar to recombination activating protein 1 [*Conilurus penicillatus*, E value 2.9]	1.8 / 3.8	1.4	2.5	+	
yokG	unknown; similar to DNA repair protein [*Tenacibaculum* sp. MED152, E value 0.71]	1.2 / 2.6	1.8	1.6		
msrA	peptidyl methionine sulfoxide reductase	2.2 / 4.9	1.7	2.5		
pbuX	xanthine permease	1.5 / 3.7	1.5	2.2		
ypsC	unknown; similar to N^6-adenine-specific DNA methylase [*B. clausii* KSM-K16, E value 1e-144]	1.8 / 5.8	1.8	2.4		
recU	DNA repair, homologous recombination and chromosome segregation	2.1 / 4.7	1.8	2.1		
ypmB	unknown; similar to nucleoprotein / polynucleotide - associated enzyme [*Escherichia coli* O157:H7 EDL933, E value 0.23]	1.4 / 2.5	1.1	1.3		
ndk	nucleoside diphosphate kinase	2.1 / 3.3	2.4	1.7		
mtrA	GTP cyclohydrolase I	1.9 / 2.7	1.4	2.0		
ypuA	unknown; similar to DNA topoisomerase I [*Mycoplasma gallisepticum* R, E value 4.3]; DNA polymerase V, putative [*Neosartorya fischeri* NRRL 181, E value 5.6]	1.2 / 3. 0	1.8	2.4		
yqkC	unknown; similar to hypothetical protein BSU23650 [*B. subtilis* subsp. *subtilis* str. 168, E value 2e-40]	1.4 / 2.9	1.6	2.3		
yqkB	unknown; similar to molybdopterin oxidoreductase [*Alkalilimnicola ehrlichei* MLHE-1, E value 5.6]	1.1 / 2.6	2.1	1.5	+	
yqkA	unknown; similar to acetyltransferase YqkA [*B. licheniformis* ATCC 14580, E value 2e-82]	1.2 / 4.0	1.7	2.2	+	
yqjZ	unknown; similar to antibiotic biosynthesis monooxygenase [*Pseudomonas fluorescens* PfO-1, E value 3e-31]	1.2 / 6.7	1.8	1.2	+	MMC, UV
yqjY	unknown; similar to acetyltransferase, GNAT family [*B. anthracis* str. Ames, E value 5e-40]	1.2 / 4.3	2.2	2.4	+	MMC, UV
yqjX	unknown; similar to similar to YolD homolog [*B.* sp. NRRL B-14911, E value 1e-04]	1.8 / 3.6	1.1	1.8	+	MMC, SA, UV
yqjW	unknown; similar to DNA polymerase IV 2 (Pol IV 2), ATP/GTP-binding protein (ImpB/MucB/SamB family) [*B. halodurans* C-125, E value 1e-142]; DNA-damage repair protein [*Oceanobacillus iheyensis* HTE831, E value 1e-81]	2.2 / 9.0	2.3	3.4	+	MMC, SA, UV
yqiZ	unknown; similar to amino acid ABC transporter, ATP-binding protein [*B. cereus* ATCC 10987, E value 4e-94]	2.1 / 6.7	1.5	2.2	+	HS
yqiW	unknown; similar to uroporphyrin-III C-methyltransferase [*Ralstonia eutropha* JMP134, E value 0.89]	1.7 / 3.6	2.3	3.4		
yqiB	unknown; similar to exodeoxyribonuclease VII large subunit [*Geobacillus stearothermophilus*, E value 1e-136]	2.0 / 8.8	1.4	3.1		
pbpA	penicillin-binding protein 2A (spore outgrowth)	1.4 / 2.8	2.2	2.4		
yqfS	unknown; similar to (AP, apurinic apyrimidinic) endonuclease IV [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-169]	2.9 / 6.2	2.1	1.6		
yqfR	unknown; similar to helicase, C-terminal: DEAD / DEAH box helicase, N-terminal [*B. cereus* subsp. *cytotoxis* NVH 391-98, E value 1e-139]	2.0 / 2.8	1.7	1.8		

yqfN	unknown; similar to S-adenosylmethionine (SAM)-dependent methyltransferase [*B. clausii* KSM-K16, E value 1e-44]	1.2 / 3.3	1.9	2.5		
dnaG	DNA primase	1.3 / 3.8	1.4	2.7		
cdd	cytidine / deoxycytidine deaminase	1.2 / 2.5	1.6	1.7		
yqfA	unknown; similar to ATPase involved in DNA repair [*Cytophaga hutchinsonii* ATCC 33406, E value 3.1]	1.4 / 5.8	2.0	2.8		
dnaJ	heat-shock protein (activation of DnaK)	1.4 / 2.7	2.3	3.0		HS
phrE	phosphatase (RapE) regulator	1.2 / 3.0	2.5	2.7		
yqbB	unknown; similar to hypothetical protein BSU26170 [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-148]	1.3 / 2.5	1.5	2.1		
yrkI	unknown; similar to redox protein regulator of disulfide bond formation-like [*Chlorobium chlorochromatii* CaD3, E value 1e-10]	1.5 / 3.1	1.9	1.8		
yrkH	unknown; similar to hydroxy-acylglutathione hydrolase [*B. cereus* ATCC 14579, E value 1e-118]	1.8. / 2.6	1.7	1.6		
yrkF	unknown; similar to UBA/THIF-type NAD/FAD binding fold [*Rubrobacter xylanophilus* DSM 9941, E value 2e-15]	1.5 / 5.2	1.7	2.4		
yrkE	unknown; similar to FAD-dependent pyridine nucleotide-disulphide oxidoreductase [*C. cellulolyticum* H10, E value 5e-14]	1.8 / 7.0	1.8	2.5		
yrhH	unknown; similar to SAM dependent methyltransferase YrrH [*B. licheniformis* ATCC 14580, E value 1e-23]	2.4 / 2.5	2.0	1.9		
yrrK	unknown; similar to Holliday junction resolvase-like protein [*B. subtilis* subsp. *subtilis* str. 168, E value 8e-72]	2.0 / 13.4	1.6	2.7		
yrzL	unknown; similar to hypothetical protein BSU27400 [*B. subtilis* subsp. *subtilis* str. 168, E value 8e-44]	1.8 / 4.4	2.0	2.7		
yrvE	unknown; similar to single-strand DNA-specific exonuclease (RecJ) [*B. licheniformis* ATCC 14580, E value 0.0]	2.0 / 7.9	1.3	2.0		
ruvB	Holliday junction DNA helicase	1.8 / 6.5	1.7	3.1	+	MMC, UV
radC	probable DNA repair protein	2.1 / 9.4	2.5	4.3		
clpX	ATP-dependent Clp protease ATP-binding subunit (class III heat-shock protein)	1.9 / 4.8	2.1	2.0		GS, HS
ysnB	unknown; similar to phosphoesterase, [*B. anthracis* str. Ames, E value 9e-40]	1.6. / 3.0	1.7	1.7		
ysmA	unknown; similar to thioesterase superfamily [*B. weihenstephanensis* KBAB4, E value 2e-36]	1.4 / 5.5	1.6	2.4		
ytxH	unknown; similar to general stress protein [*B. halodurans* C-125, E value 8e-16]; MutS 2 protein [*Thermoanaerobacter ethanolicus* ATCC 33223, E value 0.17]; recombination and DNA strand exchange inhibitor protein [*Thermoanaerobacter tengcongensis* MB4, E value 0.23]	1.8 / 5.8	1.7	1.4		GS, HS
yubC	unknown; similar to cysteine dioxygenase, type I [*Myxococcus xanthus* DK 1622, E value 1e-09]	1.2 / 4.7	1.8	2.0		
yuiH	unknown; similar to oxidoreductase (sulfite oxidase) YuiH [*B. licheniformis* ATCC 14580, E value 2e-85]	1.7 / 3.2	2.4	3.2		
yumC	unknown; similar to pyridine nucleotide-disulphide oxidoreductase [*B. anthracis* str. Ames, E value 1e-144]	1.3 / 6.4	1.8	2.0		
mrgA	metalloregulation DNA-binding stress protein	2.2 / 5.0	1.9	2.4		PS, SA^R
bdbD	thiol-disulfide oxidoreductase	1.8 / 2.6	1.8	2.0		
gapA	glyceraldehyde-3-phosphate dehydrogenase	1.1 / 2.6	1.8	1.7		
yvcI	unknown; similar to mutator MutT protein (MutT/Nudix family) [*B.* sp. NRRL B-14911, E value 2e-63]	1.4 / 3.9	2.2	3.0		

uvrA	excinuclease ABC (subunit A)	2.1 / 5.6	1.4	1.3	+	MMC, PS, UV
uvrB	excinuclease ABC (subunit B)	3.0 / 6.4	1.8	1.2	+	MMC, PS, UV
tagC	possibly involved in polyglycerol phosphate teichoic acid biosynthesis (DinR-binding site; DNA-damage inducible; *dinC*)	6.1 / 21.3	1.4	2.8	+	MMC, PS, UV
atpG	ATP synthase (subunit gamma)	2.0 / 3.2	1.4	1.7		
atpB	ATP synthase (subunit a)	3.1 / 2.8	1.3	1.7		
pyrG	CTP synthetase	1.4 / 2.7	1.7	2.0		
ywjD	unknown; similar to UV-endonuclease (UvsE / Uve1 / UvdE family) [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-109]	3.9 / 12.4	3.6	5.7		AG
thiD	possible phosphomethylpyrimidine kinase	1.8 / 2.9	1.5	2.0		
yxlH	unknown; similar to DNA polymerase III, alpha subunit [*Saccharophagus degradans* 2-40, E value 1e-13]	1.7 / 4.1	1.9	2.3		HS
nupC	pyrimidine-nucleoside transport protein	1.2 / 3.3	1.9	2.1		
ahpC	alkyl hydroperoxide reductase (small subunit)	1.9 / 2.6	2.5	1.7		GS, PS
purA	adenylosuccinate synthetase	1.7 / 7.8	2.0	3.0		
dnaC	replicative DNA helicase	2.7 / 6.3	1.6	2.7		
ssb	single-strand DNA-binding protein	8.3 / 17.0	4.3	3.6		MMC, UV
yyaF	unknown; similar to GTP-dependent nucleic acid-binding protein EngD [*B. licheniformis* ATCC 14580, E value 1e-177]	1.7 / 3.6	2.1	3.4		

[a]Genes listed by map position. The underlined genes are part of the prophage SPβ chromosome in the *B. subtilis* 168 genome (Kunst et al., 1997). [b]Function according to SubtiList-server (http://genolist.pasteur.fr/SubtiList). The function of the protein encoded is given if it has been supported by genetic or biochemical data. Otherwise the nearest homolog of the protein encoded is listed (*B*. for *Bacillus*; *C*. for *Clostridium*). The expect values (E values) given in the parentheses were obtained with BLAST search and the respective Smith-Waterman report. Detailed information on the respective "*y"*-gene was obtained with bibliographic search against several protein- and genomic databases such as Nrport, SWISS-PROT, GenePept, PIR, NRL3D, JProGo and PRODORIC. Database similarity searches were made using BLAST (Altschul et al., 1997) at the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST; see chapter 2.4, 2.4.6). In cases where the best homolog has not been characterized yet, the next possible homolog with known function is given instead. [c]All listed fold induction values are mean values of the ratios computed from eight separate slides for each genotype (n = 4 independent experiments for all treatments i.e. UV-C, UV-A, ionizing radiation and high vacuum). Ratio induction of irradiated w.t.: wild-type *B. subtilis* 168 (DSM 402), recombination-mediated repair: *recA*-mutant strain *B. subtilis* 168 (WN463), nucleotide excision repair / spore photoproduct lyase *uvrB*/*splB*-double mutant strain *B. subtilis* 168 (TKJ6312) *versus* the respective non-irradiated control. A 2-fold cutoff was applied for all experiments, i.e., only effects that were ≥ 2.5-fold change were considered significant and are reported. The significance of differences was assessed by variance analysis (ANOVA and Student's *t*-test). Values were analyzed in multigroup pairwise combinations, and differences with P values of ≤ 0.005 were considered statistically significant. [d]RecA-LexA-regulated genes in *B. subtilis* 168 with LexA-binding site according to Au et al. (2005) and Goranov et al. (2006). [e]Used abbreviation for the respective stimulon (in their response to vegetative cells of *B. subtilis*): UV 254-nm ultraviolet radiation (Au et al., 2005; Goranov et al., 2006), MMC mitomycin C (Au et al., 2005; Goranov et al., 2006); HS heat shock (Helmann et al., 2001); PS peroxide stress (Helmann et al., 2003); AG anaerobic growth (Ye et al., 2000); SA high salinity and SA^R repressed by high salinity (Steil et al., 2003) and GS general stress response (EtOH, heat and salt) (Petersohn et al., 2001).

For the functional interpretation of the transcriptional activities after 30 and 60 min germination, overrepresented groups of functional related genes (according to gene ontology groups) were identified by using bibliographic database search (see 2.4.4, 2.4.5 and 2.4.6) e.g. adapted and modified from the JProGo program suite (Scheer et al., 2006). Seven individual gene ontology groups (GO) are listed in Table 3-9 and the most prominent functional categories in relation to their gene expression will be discussed.

Table 3-9 Highly UV-C induced genes and their functional affiliation in 7 gene ontology groups (GOs)[a]

GO 1. DNA repair and modification/restriction

wild-type: *dinB, lexA, radC, tagC (dinC), uvrA, uvrB, uvrX, yacK (disA), yerH•, yjcD, yjlC•, ykoQ, ykuK, ylbH, ymaB, yobH, yoeB•, yokG•, yolB•, yomG, yoqJ•, yorH•, yozK, ypsC, yqfA•, yqfS, yqiB, yqjW, yvcI, ywjD*

recA: *radC, ykoQ, ymaB, yolB, yoqJ•, ywjD*

uvrB/*splB*: *dinB, lexA, radC, tagC (dinC), uvrX, ylbH, yqfA•, yqiB, yqjW, yvcI, ywjD*

GO 2. DNA recombination

wild-type: *recA, recF, recU, ruvB, yloC, yneB, yocI, yokH•, yolA•, yomM•, yomV•, yonX•, yorK, yrvE, yrrK*

recA: -

uvrB/*splB*: *recA, ruvB, yloC, yokH, yolA•, yrrK*

GO 3. DNA replication

wild-type: *dnaA, dnaC, dnaG, dnaX, ssb, yomH•, yopJ•, yorC•, yorJ•, yorL, yorS•, yosH•, yotL•*

recA: *ssb*

uvrB/*splB*: *dnaC, dnaG, dnaX, ssb, yomH•, yopJ•*

GO 4. DNA packaging and segregation

wild-type: *gyrB, ymcA•, yolD•, yozL•, ypuA•, yqfR, yqjX*

recA: -

uvrB/*splB*: -

GO 5. Nucleotides, nucleobases, nucleotide binding and nucleic acid metabolism

wild-type: *adk, cdd, gmk, mtrA, ndk, nupC, pbuG, pbuX, purA, purK, purR, pyrG, pyrH, thiD, yabR, yazB, yjcR, ykvM, ylaG, ylaH, ylxM, yobL•, yocG, yomO•, yomU•, yorG, yorS, ypmB•, yrkE, yyaF*

recA: *yocG*

uvrB/*splB*: *purA, ylaH, yorG, yrkE, yyaF*

GO 6. Adaptation, detoxification and response to stress (UV-C stimulus)

wild-type: *aadK, ahpC, bdbD, clpE, clpX, clpY, dnaJ, mrgA, msrA, ylzA•, yqjZ, yqkB•, ytxH, yuiH, yumC*

recA: *ahpC, clpE*

uvrB/*splB*: *clpE, dnaJ, msrA, yuiH*

GO 7. Other functions

GO 7.1 Germination and cell envelope

wild-type: *murG, pbpA, ykvT, yocH*

recA: *yocH*

uvrB/*splB*: *murG, yocH*

GO 7.2 Bioenergetics

wild-type: *atpB, atpG, ndhF*

recA: -

uvrB/*splB*: -

GO 7.3 Other functions, or/and unknown and hypothetical proteins

wild-type: *frr, gapA, infA, phrE, prsA, sigW, yloV, ymdA, yncD, yocJ, yolF•, yorF, ypbB, yqfN, yqiW, yqiZ, yqjY, yqkA, yqkC, yrhH, yrkF, yrkH, yrkI, yrzL, ysmA, ysnB, yubC*

recA: *phrE*

uvrB/*splB*: *phrE, yncD, yorF, yqfN, yqiW, yrzL*

• Gene with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). For details on the gene function, see Table 3-8. The gene ontology groups 1-7 (GO), represented in Table 3-9 (and other related Tables i.e. 3-14, 3-21 and 3-23), include several different GO nodes (according to Liu et al., 2001; Muench et al., 2003; Scheer et al., 2006) to verify the gene expression of treated spores during spore germination and to display an unified visualization on the basis of functional interpretation of biological processes, molecular functions and cell component(s). The GO 1. – 7. are combined as followed described: GO 1. (DNA repair GO: 0006281, SOS response GO: 0009432, base-excision repair GO: 0006284, nucleotide-excision repair GO: 0006289); GO 2. (DNA recombination GO: 0006310); GO 3. (DNA replication GO: 0006240); GO 4. (DNA packaging GO: 0006323, DNA

segregation GO: 0007059), GO 5. (nucleobase, nucleoside, nucleotide and nucleic acid metabolic process GO: 0006139, nucleotide biosynthetic process GO: 0009165, nucleotide metabolic process GO: 0009117, pyrimidine nucleotide biosynthetic process GO: 0006221, pyrimidine nucleotide metabolic process GO: 0006220, purine nucleotide biosynthetic process GO: 0006164, purine nucleotide metabolic process GO: 0006163, nucleobase, nucleoside, nucleotide kinase activity GO: 0019205, DNA metabolic process GO: 0006259); GO 6. (response to stress GO: 0009650, response to stimulus GO: 0050896, response to DNA damage stimulus GO: 0050896, response to external stimulus GO: 0009605, response to toxin GO: 0009636, oxidoreductase activity GO: 0016491) and GO 7. (spore germination GO: 0009847, cell envelope GO: 0030313, ATP metabolic process GO:0046034, ATP regeneration GO: 0006759). [a] Only genes with a fold induction value ≥ 2.5 (after 60 min germination) are reported.

Among these highly up-regulated transcripts many genes have described functions that may be directly involved in DNA repair, recombination and replication processes (Table 3-9, gene ontology groups GO 1.-3.) and genes involved in nucleotide stability and integrity (GO 5.).

Typical DNA repair genes such as the two DNA damage-inducible genes *dinB* and former *dinC* (tagC), the NER (nucleotide excision repair) exonucleases *uvrA* and *uvrB*, the probable DNA repair protein *radC* and *recA*, the multifunctional protein involved in the homologous recombination (and recombination-mediated repair) and the single-strand DNA-binding encoding gene *ssb* showed significant increased fluorescent values, as can seen in Table 3-8. Twelve different DNA polymerases, six DNA polymerases type III, DNA polymerases directly involved in the DNA replication and elongation: *dnaX*, *yosH*, *yorS*, *yorL*, *yorC* and *yomH*; five DNA polymerase type IV (DinB; damage-inducible): *uvrX*, *yobH*, *yozK*, *yqjW* and *yxlH* and one DNA polymerase type V (UmuC-like, SOS-inducible): *ypuA* were also significantly up-regulated after 60 min germination (Table 3-8).

Rec-genes like *recA*, *recF* (with single-strand, ssDNA and double-strand, dsDNA binding activity) and *recO* (assists *recF* and *ssb* in the RecA filament formation) that are likely to be also implicated in SOS-induced DNA repair processes (recombination-mediated repair) were also significantly induced by UV-C irradiation. In addition to the known *rec*-genes, seven genes which are expected to be involved in the homologous recombination (and recombination-mediated repair) were significantly induced: *ruvB* (a known Holliday junction DNA helicase) and *yrrK* (a predicted Holliday junction resolvase like encoding gene of *B. subtilis* subsp. *subtilis* str. 168), *yneB* a probable site-specific resolvase/recombinase (similar to *B. cereus* ATCC 14579), two *recJ*-like (single-stranded-DNA

specific exonuclease) genes: *yorK* (similar to *Staphylococcus epidermidis* RP62A) and *yrvE* (a *B. licheniformis* ATCC 14580 homolog), and two ATP-depending DNA helicases *yocI* (a *B. cereus* ATCC 14579 homolog) and *yloC* (similar to *Saccharophagus degradans*) (Table 3-9, GO 2.).

Further on, two "*y*"-genes (*yjcD* and *ywjD*) with high sequence similarity to an UV-responsive UvrD-like ATP-depending DNA helicase (similar to *B. clausii* KSM-K16) and an UV-endonuclease (UvsE/Uve1/UvdE, similar to *B. thuringiensis* servor *israelensis* ATCC 35646) were significantly up-regulated in the wild-type strain (> 9), while for HR- and NER/SP lyase- deficient strains the induction ratio was below the 2-fold cutoff and in the case of *yjcD*, 5- and 2-fold weaker, respectively for *ywjD* for the NER/SP lyase- and HR-mutant strains. That suggests that *yjcD* has a *recA*-dependent induction, which is in good agreement with previous observations on the *uvrD/rep* DNA helicase of *E. coli*. Veaute et al. (2005) reported that this *uvrD/rep* DNA helicase acts as RecA nucleoprotein filament remover. Two MutT/nudix families (known to be involved in the oxidative stress response; Kang et al., 2003): *yvcI* (with sequence homology to *Bacillus* sp. NRRL B-14911) and *ymaB* (homolog to *Staphylococcus epidermidis* RP62A) were significantly induced.

Thirty genes involved in the nucleotide binding and nucleic acid metabolism were found to be up-regulated after UV-stress and 60 min germination, several nucleotide kinases and nucleotide triphosphates (NTPs) binding encoding genes e.g. *yyaF* (a predicted GTP-dependent nucleic acid-binding protein, EngD-like similar to *B. licheniformis* ATCC 14580) were strongly induced after 1 h germination (Tables 3-8 and 3-9).

Among the main DNA repair mechanism pathways, a large group of kinases, involved in nucleotide metabolism (pyrimidine metabolism and purine metabolism) showed a higher level of gene activation than kinases of the non-irradiated samples, particularly *adk* (adenylate kinase (enzyme classification number EC: 2.7.4.3)), *gmk* (guanylate kinase (EC: 2.7.4.8)) and *ndk* (nucleoside diphosphate kinase (EC: 2.7.4.6)). These kinases are enzymes that transfer phosphate groups from high-energy donor molecules, such as ATP, to specific target molecules (substrates), in an ATP phosphorylation process. It is known that dormant spores contain a large depot of 3-PGA (3-phosphoglyceric acid) that is rapidly

utilized in the first minutes of spore germination to generate ATP. Over 90 % of the 3-PGA depot was lost by 30 min after initiation of germination (Setlow et al., 2001).

According to this data and by knowing that DNA repair involves engery-dependent processes, these kinases are closely linked to all four DNA nucleotides (dATP, dTTP, dCTP, dGTP) and their phosphorylation. This implicates their important role in the essential replacement/substitution of radiation-damaged nucleotides e.g. oxidized nucleobases (i.e. 8-oxo-7,8-dihydro-2'-deoxyguanosine; Douki et al., 2006) and the initiation as well as the assembling of new DNA strand fragments for a potential gap filling and/or new DNA strand synthesis.

Fourteen up-regulated genes were belonging to detoxification processes e.g. *mrgA* (hydrogen peroxide resistance) and *msrA* (response to oxidizing agents), or responses to stress e.g. *ytxH* (a general stress protein with a high sequence homology to *B. halodurans* C-125) and *yuiH*, a putative oxidoreductase - similar to a sulfite oxidase of *B. licheniformis* ATCC 14580 (Table 3-9, GO 6.). Further on, two yet unknown genes were found to be up-regulated that are involved in spore-cortex lytic processes (*ykvT*, similar to *Geobacillus kaustophilus* HTA426) and cell wall-binding e.g. *yocH* an *N*-acetylmuramoyl-*L*-alanine amidase (homolog to *Oceanobacillus iheyensis* THE831). In addition to the induced nucleotide binding proteins 2 genes involved in the ATP synthesis (*atpB* and *atpG*) were up-regulated (GO 7.1, see Table 3-9).

Deficiencies in the *recA* (homologous recombination and recombination-mediated repair), *splB* (spore photoproduct lyase) and *uvrB* (nucleotide excision repair) led to dramatic decrease in the removal of UV-C induced DNA damage during spore germination (Table 3-6) and to a weaker (less specific) response in the global gene expression (as can seen in Table 3-8), mostly affected by the disruption and malfunction of these key enzymes i.e. *recA* (its general impact in the single- and double-strand break repair) and *splB* (its unique involvement the repair of the UV-induced SP). The three highest up-regulated genes in these two repair-deficient strains were *ssb, ykoQ* (a putative DNA repair exonuclease, similar to *B. cereus* E33L) and *ywjD* for the *recA*-deficient strain, and *recA, radC* and *ywjD* for the *uvrB*/*splB* mutant strain. This leads to the assumption that *ywjD* is a *recA*-independent UV-endonuclease.

In order to get further confirmation of the validity of the DNA microarray data, the highly induced putative UV-endonuclease *ywjD* (12.4 after UV-C and 15.1 after UV-A irradiation (see chapter 3.3.3, Table 3-13) and 60 min germination) was selected for RT-PCR experiments (as described in 2.4.7). Fig. 3-10 shows the DNA bands of the amplified cDNA after RT-PCR. The rows were loaded as follows: row 1: UV-C irradiated (wild-type), 2: non-UV-C irradiated, 3: X-rays irradiated (ionizing radiation), 4: non-ionizing radiation exposed, 5: UV-(A+B) irradiated, 6: non-UV-(A+B) irradiated, 7: UV-A irradiated, 8: non-UV-A irradiated, 9: UV-C irradiated (major SASPs-deficient spores, α/β-SASPs: *sspA sspB*), 10: non-UV-C irradiated (*sspA sspB*) and M: standard DNA marker (Hyper Ladder IV, Bioline GmbH, Luckenwalde, Germany). The amplified cDNA band for the estimated RT-PCR product was in the expected product size (258 bp).

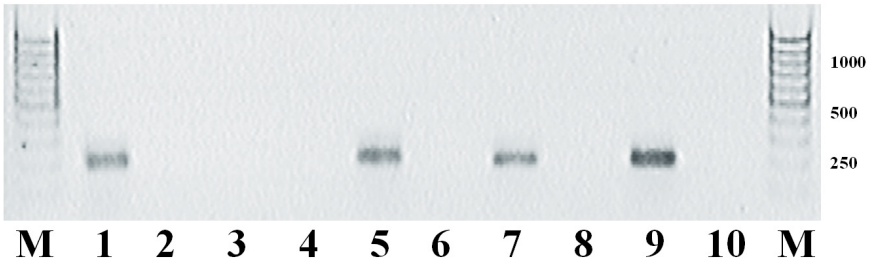


Fig. 3-10 RT-PCR analysis of UV and X-rays exposed (1, 3, 5, 7, 9) and non-irradiated (2, 4, 6, 8, 10) wild-type and SASPs-deficient *B. subtilis* 168 *ywjD* - transcript after 60 min germination.

Similar cDNA band patterns were obtained for *ykoQ*, *yjcD* (a probable *uvrD*-like DNA helicase), *yobH* and *uvrX* (two predicted UV damage-repair error-prone polymerases) from RNA samples isolated out of UV-C irradiated and of non-irradiated 60 min-germinating wild-type *B. subtilis* 168 spores (for functional characterization and sequence homologies see Tables 3-8 and 3-9). Fig. 3-10 can be seen as standard example for gene verification via RT-PCR.

3.3 Responses to UV-A radiation

Spores of wild-type, three DNA repair- or α/β-type SAPSs-deficient *B. subtilis* strains were exposed to UV-A (320 - 400 nm) irradiation, the major part of the terrestrial UV climate, as described in 2.2.3.2. Mutant spores of following DNA repair mechanisms were tested: recombination-mediated repair (RR; *recA*), nucleotide excision repair /SP lyase (*splB uvrB*) and RR/NER/SP lyase (*recA splB uvrB*).

3.3.1 Survival of UV-A irradiated *B. subtilis* spores

Spore inactivation kinetics and photoproduct inductions curves were assessed in the same samples, similar to the experiments with UV-C (3.2). The UV-A survival curves (2.5, 2.5.1) of the spores were either exponential (for the SASPs- and the DNA repair deficient strains) or showed a slight shoulder (for wild-type spores), as indicated by n > 1. (Fig. 3-11 and Table 3-10). The survival response of the wild-type *B. subtilis* spores is in good agreement with previous published data (Hullo et al., 2001; Moeller et al., 2005). From the fluence-effect curves of inactivation (Fig. 3-11), key survival characteristics were derived (Table 3-10).

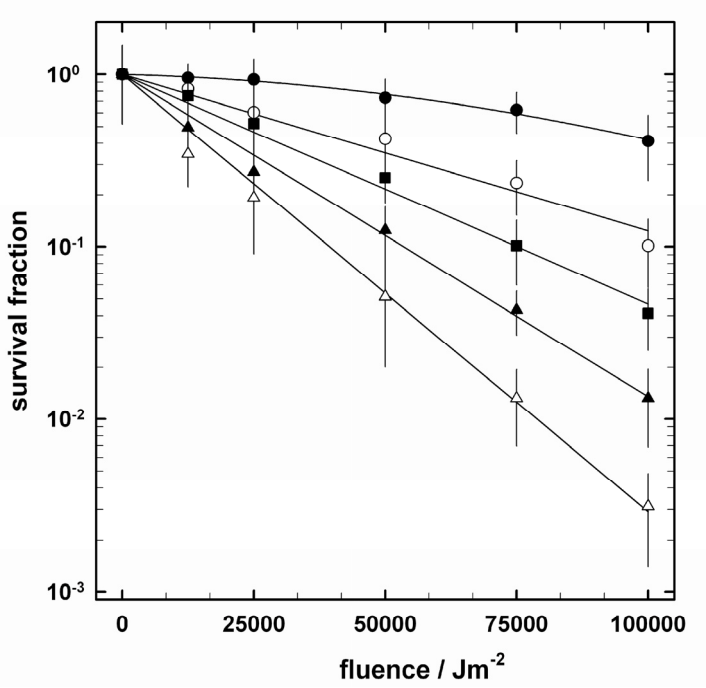


Fig. 3-11 Fluence-survival curve *B. subtilis* wild-type (solid circles), *recA* (open circles), *sspA sspB* (solid squares), *splB uvrB* (solid triangles) and *recA splB uvrB* (open triangles) after exposure to UV-A radiation. Data are averages +/- standard deviation ($n = 4$).

The data show that spore UV-A resistance decreased in the order (wild-type > *recA* > *sspA sspB* > *splB uvrB* > *recA splB uvrB*) similar to the trend observed after UV-C irradiation. To obtain 90 % inactivation of the wild-type spores, they had to be irradiated with approximately 280 kJ/m² UV-A. This value is roughly 1170-times higher than the fluence required for UV-C application, which is in good agreement with previous data of UV-A irradiated spores and the action spectra for DNA inactivation (Moeller et al., 2005; Horneck and Rabbow, 2007). Examination of the RF values (Table 3-10) shows that among the repair mechanisms, the recombination-mediated repair (*recA*) was the least

efficient for repairing UV-A damage induced in spores, but slightly weaker response (about 40 %) compared with the UV-C data. The combined DNA photoproduct repair mechanisms SP lyase and NER had the highest repair efficiency shown by the wild-type response and their reverse repair answer of the respective tested mutant spores (Table 3-10 and Fig. 3-11). .

Table 3-10 Survival curve characteristics after UV-A irradiation (320-400 nm)

UV-A (320-400 nm)	F_{10} (kJ/m²)	F_0 (kJ/m²)	F_q (kJ/m²)	IC (m²/J)
B. subtilis (wild-type)	277.1 ± 41.0[a]	124.8 ± 22.9[a]	9.0 ± 2.1	$(8.59 \pm 1.44)\times10^{-6}$
sspA sspB	74.9 ± 13.3	34.5 ± 8.7	3.3 ± 0.9	$(3.21 \pm 0.65)\times10^{-5}$
recA	108.3 ± 32.7	48.7 ± 13.2	3.8 ± 1.4	$(2.24 \pm 0.33)\times10^{-5}$
splB uvrB	52.8 ± 18.5	21.3 ± 4.6	n.d.	$(4.16 \pm 0.28)\times10^{-5}$
recA splB uvrB	37.9 ± 21.2	14.5 ± 2.3	n.d.	$(5.58 \pm 0.59)\times10^{-5}$

UV-A (320-400 nm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.28 ± 0.11	1	0.943	
sspA sspB	0.95 ± 0.14	0.37 ± 0.14	0.995	0.019
recA	0.97 ± 0.22	0.48 ± 0.17	0.979	0.013
splB uvrB	0.79 ± 0.08	0.27 ± 0.09	0.995	0.007
recA splB uvrB	0.72 ± 0.13	0.23 ± 0.11	0.968	0.006

n.d. not determinable. RF = The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis.* [a]Value obtained by extrapolation. $P \leq 0.05$ = significant difference.

3.3.2 Induction and repair of DNA photolesions in UV-A irradiated *B. subtilis* spores

Whereas several data exist already on the induction of Guanine photoproducts, e.g. 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), after UV-A irradiation (Cadet et al., 2002; 2005), which are caused by indirect effects via induced radicals, this work concentrates on the direct effects in the DNA of UV-A irradiated spores, i.e. photoproducts involving pyrimidines. Immediately after UV-A irradiation DNA was extracted out of wild-type, *recA* or α/β-type SASPs-deficient (*sspA sspB*) spores and analyzed for the induction of DNA bipyrimidine photoproducts, as described in 2.1.7 and 2.3.1. In Fig. 3-12 a fluence-depending increase of the photoproducts is shown for wild-type (Fig. 3-12A) and SASPs-deficient (Fig. 3-12B) spores. The spore photoproduct (SP) was the major photoproduct (> 95

%) in the wild-type spores, SP was still the major lesion but in lower quantity (about 50 % of the total amount) in SASPs-deficient spores. In spores of the latter strain the cyclobutane pyrimidine dimers (CPD) of thymine-thymine (CPD TT) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PP) of thymine-cytosine (6-4 TC) were formed with 10-fold higher efficiency than in the wild-type spores. These differences in the distribution of the bipyrimidine photoproducts in wild-type compared to SASPs-deficient spores are similar to those observed in spores of both genotypes after UV-C irradiation (3.2.2 Fig. 3-7, Table 3-6). The efficiency of PP formation by UV-A was about 785-times lower than by UV-C (calculated on the ratio of the average induction, see Tables 3-6 and 3-11, for all strains tested)

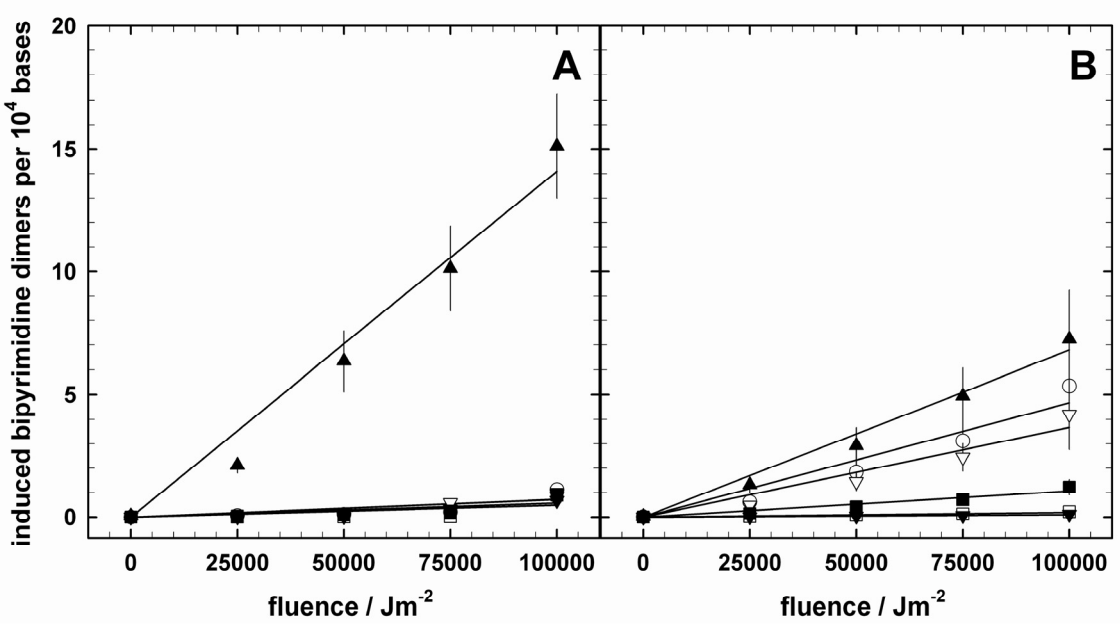


Fig. 3-12 Fluence-effect curves of the induction of DNA bipyrimidine photoproducts following UV-A irradiation of spores (**A**) small, acid-soluble spore proteins (SASP) wild-type (*B. subtilis* 168 wild-type) and (**B**) α/β-type SASP deficient (*sspA sspB*); CPD CT (solid triangles down), CPD TC (solid squares), CPD TT (open circles), 6-4 TC (open triangles down), 6-4 TT (open squares) and SP (solid triangles up). Data are reported as averages and standard deviations (n = 3).

A fluence of 100 kJ/m^2 of UV-A radiation induced approximately 12000 DNA bipyrimidine photoproducts in a single spore chromosome. In Table 3-11 the detailed distribution of induced PP is shown for wild-type, *recA* and *sspA sspB* spores. It shows – similar to the results from UV-C radiation - that the total yield of PP is statistically identical for the differents strains tested; however, in SASPs-

deficient spores a dramatic increase in CPDs and 6-4PPs formation was found compared to α/β-type SASP-wild-type spores.

Table 3-11 Yields of photolesions in *Bacillus* sp. spores exposed to 100 kJ/m² UV-A radiation.

Strain	SP (TDHT)	CPD TC	CPD TT	6-4 TC
B. subtilis (wild-type)	$(1.5 \pm 0.2)\times10^{-4}$	$(3.6 \pm 0.6)\times10^{-6}$	$(4.7 \pm 1.1)\times10^{-6}$	$(5.2 \pm 0.6)\times10^{-6}$
sspA sspB	$(6.3 \pm 1.3)\times10^{-5}$	$(8.9 \pm 1.4)\times10^{-6}$	$(3.9 \pm 0.5)\times10^{-5}$	$(3.1 \pm 0.7)\times10^{-5}$
recA	$(1.1 \pm 0.2)\times10^{-4}$	$(2.8 \pm 0.6)\times10^{-6}$	$(3.9 \pm 0.7)\times10^{-6}$	$(6.0 \pm 0.4)\times10^{-6}$

Strain	CPD CT	6-4 TT	Total	total PP / 1 chromosome[a]
B. subtilis (wild-type)	$(2.9 \pm 0.6)\times10^{-6}$	$(2.5 \pm 0.9)\times10^{-6}$	$(1.6 \pm 0.2)\times10^{-4}$	$(1.3 \pm 0.2)\times10^{4}$
sspA sspB	$(7.9 \pm 2.9)\times10^{-7}$	$(1.6 \pm 0.5)\times10^{-6}$	$(1.4 \pm 0.3)\times10^{-4}$	$(1.2 \pm 0.1)\times10^{4}$
recA	$(4.0 \pm 1.7)\times10^{-6}$	$(2.4 \pm 0.5)\times10^{-6}$	$(1.3 \pm 0.4)\times10^{-4}$	$(1.1 \pm 0.3)\times10^{4}$

Data are expressed as lesions/10^4 bases per Jm^{-2}. [a]DNA PP level calculated on the chromosome size of *B. subtilis* 168 (according to the genomic information published by Kunst et al., 1997). Data are averages with standard deviations (n = 4).

For studying PP repair during germination and outgrowth of UV-A (100 kJ/m^2) irradiated, wild-type and mutant spores, the PP removal was traced as described previously in 3.2.2. In short, spores were incubated in germination medium with an *L*-alanine supplement as germination triggering agent under optimal conditions (temperature and aeration). In Fig. 3-13 repair kinetics for the wild-type spores are shown; **A**: SP repair and **B**: CPDs and 6-4PPs repair.

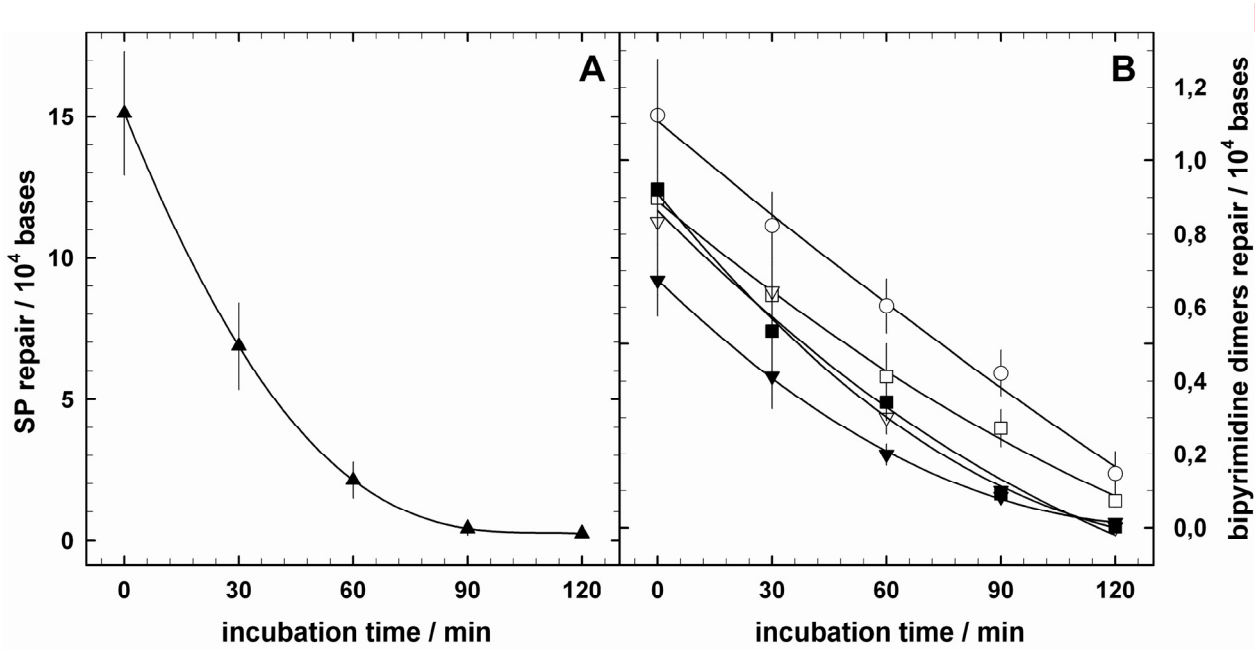


Fig. 3-13 Repair kinetics of the induced major photoproduct, the spore photoproduct SP (**A**) and of minor bipyrimidine photoproducts (**B**) CPD CT (solid triangles down), CPD TC (solid squares), CPD TT (open circles), 6-4 TC (open triangles down), 6-4 TT (open squares) in wild-type *B. subtilis* 168 spores during spore germination and outgrowth. Data are reported as averages and standard deviations (n = 3).

In Table 3-12 the efficiency of repair after 60 min of spore germination is shown, expressed as percent of UV-A induced photoproduct repaired relative to time zero of germination, for wild-type, *recA*- and SASPs-mutant spores. After 60 min germination roughly 80 % of the total UV-A induced PP were repaired in the wild-type and SASPs-deficient spores, whereas only 45 % were removed in the *recA* mutant spores.

Table 3-12 Repair of UV-A (100 kJ/m²) induced DNA bipyrimidine photoproducts after 60 min germination of wild-type and mutant *B. subtilis* spores

Strain	SP (TDHT)	CPD TC	CPD TT	6-4 TC	CPD CT	6-4 TT	Total
wild-type	78.5 ± 7.2	66.3 ± 12.2	47.5 ± 9.9	64.3 ± 19.1	69.3 ± 15.4	54.7 ± 14.1	79.7 ± 9.8
sspA sspB	76.5 ± 16.3	60.1 ± 14.0	57.1 ± 13.0	53.4 ± 9.2	47.5 ± 12.1	65.2 ± 10.7	76.3 ± 13.7
recA	44.8 ± 11.4	29.4 ± 8.2	16.3 ± 4.0	22.4 ± 5.6	23.1 ± 8.8	39.7 ± 9.6	45.3 ± 8.7

Data are expressed as percent of repaired photoproducts. Data are averages and standard deviations (n = 4).

3.3.3 Transcriptional profile of UV-A irradiated germinating *B. subtilis* spores

Wild-type and *recA*-deficient spores, both irradiated with 100 kJ/m^2 UV-A radiation, were analyzed for their global transcriptional response after 60 min germination. RNA samples extracted from non-irradiated and irradiated samples from the respective strain at equal germination time were studied in cDNA microarray-based analyses. Prior to quantification of the array data, the quality and reproducibility of the array experiment were estimated by comparing the normalized spot intensities in the scattered diagram for 60 min germinating wild-type and *recA*-deficient spores, as described previously (3.2.3). Array data from hybridization of independent samples representing the same cultivation condition always yielded high Pearson correlation coefficient with $r \geq 0.900$ (Blencke et al., 2003; Moeller et al., 2007b), as measured for wild-type (0.924) and *recA* (0.915) spores (Fig. 3-14) and mentioned previously (3.2.3).

Using a 2-fold balanced differential expression as the appropriate threshold level, 387 of the 4107 known and unknown ("*y*"-) genes were found to be up-regulated in the wild-type strain (9.4 %), whereas only 218 for the *recA*-deficient (5.3 %) spores genes were induced after UV-A irradiation during spore germination.

Table 3-13 lists the 132 up-regulated genes (with a significant ≥ 2.5-fold change), determined from the 60 min-germinated wild-type as well as *recA* spore populations. In addition for all these genes the putative LexA-binding site according to Au et al. (2005) and their response to further DNA damaging agents, e.g. mitomycin C were determined by bibliographic search (for detailed information see footnotes in Table 3-8).

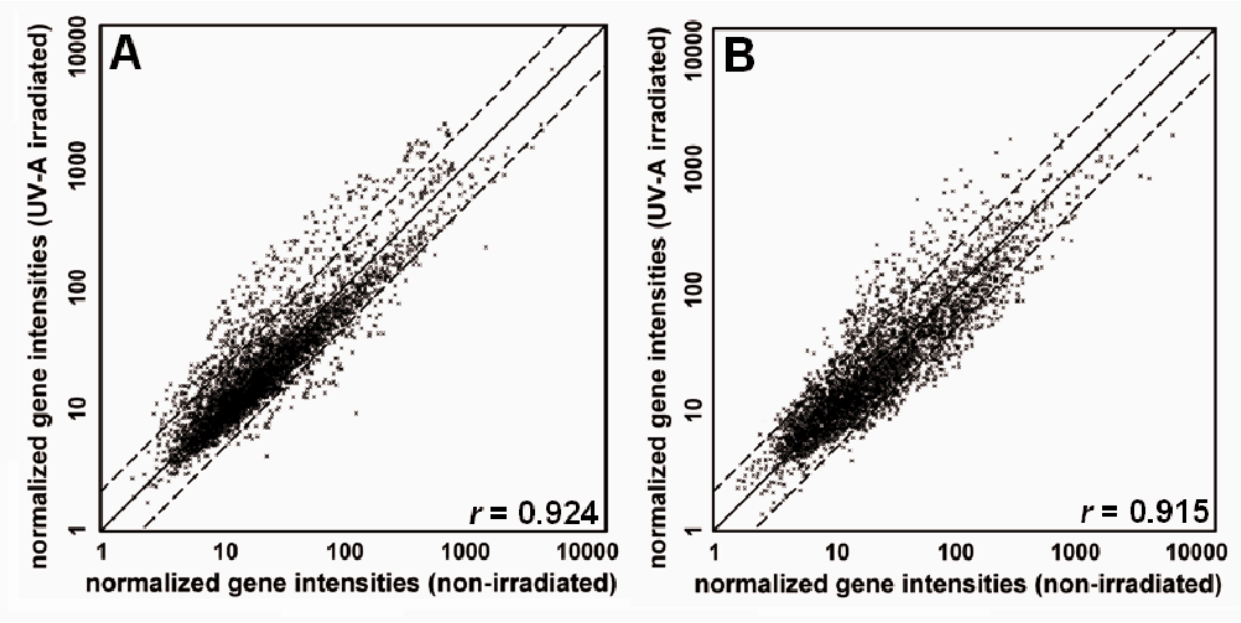


Fig. 3-14 Scatter diagrams of normalized spot intensities. Gene intensities are plotted of UV-A irradiated and non-irradiated wild-type (**A**) and *recA* (**B**) spores after 60 min germination. The significance threshold of twofold induction or repression is symbolized by the two dashed lines. Data are expressed as mean values (n = 4). r = Pearson correlation coefficient.

The majority of comparisons were performed on the base of four independent experiments; microarray data processing (fluorescent intensity differences of the ratio UV-C irradiated *versus* non-irradiated germinating spores), statistical significance data analysis and functional interpretation of the microarray data were performed as described in detail in chapter 2.4 (2.4.4 – 2.4.6).

Table 3-13 UV-A radiation-induced genes after 60 min germination[a]

Gene	Function	Fold induction		LexA-bind. site	Induced by further stimulons?
		w.t.	*recA*		
dnaA	Initiation of chromosome replication	4.7	3.1		MMC, UV
dnaN	DNA polymerase III (beta subunit)	2.4	1.6		
recF	DNA repair and genetic recombination	4.8	1.5	+	MMC, UV
yaaC	unknown; similar to conserved protein YaaC [*B. licheniformis* ATCC 14580, E value 1e-105]	2.9	2.4		
tmk	thymidylate kinase	3.4	2.0		
purr	transcriptional repressor of the purine operons	2.9	1.7		
yacK	unknown; similar to DNA-binding protein / DNA integrity scanning protein (DisA) [*B. licheniformis* ATCC 14580, E value 1e-172]	2.9	1.8		GS, HS
adk	adenylate kinase	2.6	1.3		
ybaL	unknown; similar to ATP-binding protein Mrp [*B. licheniformis* ATCC 14580, E value 1e-155]	2.5	1.7		

ybaR	unknown; similar to sulfate transporter / antisigma-factor antagonist STAS: xanthine / uracil / vitamin C permease: sulphate transporter [*B. cereus* subsp. *cytotoxis* NVH 391-98, E value 1e-137]	2.7	2.3		
sigW	RNA polymerase ECF-type sigma factor	4.1	2.4		
alkA	DNA-3-methyladenine glycosylase	4.4	3.7		
adaB	O^6-methylguanine-DNA methyltransferase	7.4	2.6		
topB	DNA topoisomerase III	4.7	2.0		
sacV	transcriptional regulator of the levansucrase gene	2.5	1.4		
yddE	unknown; similar to DNA segregation ATPase related protein [*Streptococcus pyogenes* MGAS6180, E value 1e-132]	3.3	2.7		
dinB	nuclease inhibitor	15.1	1.8	+	MMC, PS, UV
ydiM	unknown; similar to transcriptional regulator / sugar kinase [*Vibrio vulnificus* CMCP6, E value 2.8]	2.8	2.2		
ydiO	unknown; similar to DNA-methyltransferase (cytosine-specific) [*Rhodopseudomonas palustris* BisB18, E value 3e-32]	4.7	1.4	+	
ydiP	unknown; similar to DNA-methyltransferase (cytosine-specific) [*Mycobacterium* sp. KMS, E value 7e-96]	5.8	1.8	+	
pbuG	hypoxanthine / guanine permease	3.2	2.7		
pcrA	ATP-dependent DNA helicase	5.0	1.7		
yefB	unknown; similar to site-specific recombinase (resolvase family) [*Geobacillus kaustophilus* HTA426, E value 3e-55]	17.0	1.8		
yefC	unknown; similar to site-specific recombinase, resolvase family [*B. cereus* G9241, E value 2e-23]	10.8	1.4		
cotJC	polypeptide composition of the spore coat (sigma-E-controlled operon; probable undercoat localization; putative catalase activity)	2.8	2.0		
yhaZ	unknown; similar to DNA alkylation repair enzyme [*Lactobacillus sakei* subsp. *sakei* 23K, E value 4e-80]	6.0	1.4	+	MMC, PS, UV
yhaX	unknown; similar to hydrolase, haloacid dehalogenase-like family [*B. anthracis* str. Ames, E value 8e-76]	3.8	1.8		
yhaU	unknown; similar to Na^+/H^+ antiporter (sodium/hydrogen exchanger family protein) [*B. thuringiensis* str. Al Hakam, E value 1e-157]	4.1	2.2		GS, HS, SA
yhaO	unknown; similar to DNA repair exonuclease [*B. thuringiensis* str. Al Hakam, E value 3e-87]	4.6	1.7	+	MMC, PS, UV
prsA	protein secretion (post-translocation molecular chaperone)	2.7	1.5		
yirY	unknown; similar to DNA repair exonuclease [*Symbiobacterium thermophilum* IAM 14863, E value 1e-151]; ATPase involved in DNA repair [*Thermoanaerobacter tengcongensis* MB4, E value 1e-145]	4.2	2.3		
yisB	unknown; similar to restriction endonuclease [*Magnetospirillum magnetotacticum* MS-1, E value 2e-11]	2.5	2.4		
yjcD	unknown; similar to UvrD/REP family ATP-dependent DNA helicase [*B. clausii* KSM-K16, E value 1e-169]	12.4	1.8		
yjcG	unknown; similar to 2'-5' RNA ligase [*B. cereus* ATCC 14579, E value 7e-55]	2.8	1.2		
ykoQ	unknown; similar to DNA repair exonuclease / phosphoesterase [*B. cereus* E33L, E value 6e-91]	8.0	4.6		
ykoW	unknown; similar to diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s) [*Pseudomonas fluorescens* PfO-1, E value 5e-92]; sensory box/GGDEF family protein [*B. cereus* E33L, E value 6e-95]	4.6	2.0		
ykuU	unknown; similar to alkyl hydroperoxide reductase [*B. licheniformis* ATCC 14580, E value 1e-100]; 2-cys peroxiredoxin [*B. halodurans* C-125, E value 5e-90]	4.0	2.1		

ylaC	unknown; similar to RNA polymerase ECF-type sigma factor [*B. licheniformis* ATCC 14580, E value 4e-57]	3.1	2.4		
mraY	phospho-N-acetylmuramoyl-pentapeptide transferase	2.5	2.0		
murG	UDP-N-acetylglucosamine-N-acetylmuramyl-(penta-peptide) pyrophosphoryl-undecaprenol N-acetylglucos-amine transferase	3.2	1.5		GS
pyrR	transcriptional attenuation of the pyrimidine operon / uracil phosphoribosyltransferase activity	6.0	1.6		MMC, SA
gmk	guanylate kinase	2.8	1.9		
uppS	probable undecaprenyl pyrophosphate synthetase	2.8	1.1		
polC	DNA polymerase III (alpha subunit)	6.4	1.7		
pnpA	polynucleotide phosphorylase (PNPase)	2.8	2.4		
recA	multifunctional protein involved in homologous recombination and DNA repair (LexA-autocleavage)	30.1	1.2	+	MMC, PS, UV
cotE	spore coat protein (outer) (probably minor protein; may be tyrosine peroxidase)	4.5	3.0		
mutS	DNA mismatch repair (recognition)	7.4	2.6		
pksB	involved in polyketide synthesis (ATP synthase)	2.7	1.4		
ymaD	unknown; similar to organic hydroperoxide resistance protein OsmC, predicted redox protein, regulator of sulfide bond formation [*Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1, E value 2e-07]	8.1	2.7		PS
ymaB	unknown; similar to phosphoesterase [*B. clausii* KSM-K16, E value 7e-60]; MutT/nudix family protein [*Staphylococcus epidermidis* RP62A, E value 9e-34]	10.4	4.8		
lexA	transcriptional repressor of the SOS regulon	3.4	1.2	+	MMC, UV
yneB	unknown; similar to resolvase, N-terminal domain – putative recombinase [*B. licheniformis* ATCC 14580, E value 4e-90]; site-specific recombinase [*B. cereus* ATCC 14579, E value 9e-78]	9.7	1.4	+	MMC, PS, UV
yobE	unknown; similar to hypothetical protein BSU18880 [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-126]	5.4	1.9		
phrK	phosphatase (RapK) regulator	2.7	2.5		
yobH	unknown; similar to UV-damage repair protein (UvrX) [UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-108]; ImpB/MucB/SamB family protein [Bacteriophage SPBc2, E value 1e-108]	12.4	1.4	+	MMC, UV
yozL	unknown; similar to YolD homolog [*B.* sp. NRRL B-14911, E value 0.009]	4.7	1.6	+	MMC, UV
yocA	unknown; similar to transposon-related protein [*Lactococcus lactis* subsp. *lactis* Il1403, E value 9e-20]; soluble lytic murein transglycosylase related regulatory protein [*Lactococcus lactis* subsp. *cremoris* SK11, E value 5e-20]	3.0	1.8		SA
yocH	unknown; similar to cell wall-binding protein [*Oceanobacillus iheyensis* HTE831, E value 5e-46]; N-acetylmuramoyl-L-alanine amidase [*Oceanobacillus iheyensis* HTE831, E value 3e-11]	4.4	2.3		SA
yocI	unknown; similar to ATP-dependent DNA helicase (RecQ) [*B. cereus* ATCC 14579, E value 0.0]	7.4	1.6		
yocJ	unknown; similar to acyl carrier protein phosphodiesterase [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-115]	5.0	2.2		
yorS	unknown; similar to 5'(3')-deoxyribonucleotidase [*Robiginitalea biformata* HTCC2501, E value 4e-29]; DNA polymerase alpha [*Plasmodium falciparum*, E value 1.8]	4.2	1.7		
yorL	unknown; similar to DNA polymerase III, alpha subunit, truncation [*Staphylococcus epidermidis* RP62A, E value 1e-141]	3.6	1.6	+	
yorI	unknown; similar to phage replicative DNA helicase, YorI *B. subtilis* homolog [*C. acetobutylicum* ATCC 824, E value 1e-20]	2.8	1.3	+	
yorG	unknown; similar to ATP/GTP binding protein [Bacteriophage SPBc2, E value 0.0]	2.5	2.0	+	

yorA	unknown; similar to parallel beta-helix repeat [*B. weihenstephanensis* KBAB4, E value 6e-09]	3.9	2.7	
ligB	DNA ligase (ATP-dependent)	5.6	1.7	
yoqP	unknown; similar to DEAD/DEAH box helicase [*Entamoeba histolytica* HM-1:IMSS, E value 2.3]	2.5	1.4	
yoqA	unknown; similar to methyltransferase [*Rickettsia bellii* RML369-C, E value 0.77]	3.1	2.0	+
yopX	unknown; similar to diacylglycerol kinase, catalytic region [*Thermosinus carboxydivorans* Nor1, E value 2.1]	2.6	1.4	+
yopW	unknown; similar to DNA gyrase subunit B [*Streptococcus downei*, E value 0.35]	3.0	2.1	
yopT	unknown; similar to hypothetical protein BSU20770 [*B. subtilis subsp. subtilis* str. 168, E value 7e-33]	2.8	1.3	+
yopB	unknown; similar to helix-turn-helix type 3 [*Herpetosiphon aurantiacus* ATCC 23779, E value 0.028]	2.6	1.9	
yopA	unknown, similar to thiol methyltransferase 1-like [*Synechococcus elongatus* PCC 7942, E value 1e-31]	4.7	2.4	
yonV	unknown; similar to CCAAT-box DNA binding protein subunit B [*Plasmodium yoelii yoelii* str. 17XNL, E value 0.001]; DNA repair protein [*Acanthamoeba polyphaga mimivirus*, E value 0.30]	3.0	1.8	
yonG	unknown; similar to ClpX, ATPase regulatory subunit [*C.* sp. OhILAs, E value 4.7]	2.5	1.8	
yonF	unknown; similar to terminase DNA packaging enzyme, large subunit [Bacteriophage RM 378, E value 2e-04]	3.0	1.9	
yomX	unknown; similar to unknown [Bacteriophage SPBc2, E value 1e-121]; ABC transporter ATP-binding protein [*Xanthomonas axonopodis* pv. *citri* str. 306, E value 1.9]	3.7	1.5	
yomW	unknown [Bacteriophage SPBc2, E value 6e-93]	2.5	2.0	
yomS	unknown; similar to lytic exoenzyme associated with defective prophage PBSX [*B. subtilis* subsp. *subtilis* str. 168, E value 3e-12]	4.0	1.2	
yomR	unknown; similar to phage related protein [*B. licheniformis* ATCC 14580, E value 5e-35]	2.7	1.6	
yomO	unknown; similar to anticodon nuclease PrrC [*Flavobacterium johnsoniae* UW101, E value 0.43]	3.1	2.2	
yomH	unknown; similar to DNA polymerase III subunits gamma and tau [*Helicobacter acinonychis* str. Sheeba, E value 4.4]	2.5	1.8	
yomE	unknown; similar to N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase related protein [*Lactococcus lactis* subsp. *cremoris* SK11, E value 4e-09]	4.6	2.0	
uvrX	ImpB/MucB/SamB family protein [Bacteriophage SPBc2]; UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168 E value 0.0]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 1e-103]	16.1	3.0	+
volD	unknown; similar to putative chromosome segregation protein, SMC ATPase superfamily [*Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986, E value 5.1]; cyclic nucleotide-binding domain containing protein [*Tetrahymena thermophila* SB210, E value 8.7]	4.7	1.6	+
yokA	unknown; similar to resolvase, N-terminal: recombinase [*B. weihenstephanensis* KBAB4, E value 1e-40]	8.3	1.7	
yppQ	unknown; similar to methionine sulfoxide reductase B [*B. subtilis* subsp. *subtilis* str. 168, E value 4e-82]	4.1	2.7	
pbuX	xanthine permease	3.0	1.2	
recU	DNA repair, homologous recombination and chromosome segregation	7.1	1.4	

ndk	nucleoside diphosphate kinase	3.9	2.7		
hbs	non-specific DNA-binding protein HBsu	7.4	2.3	+	
yqkB	unknown; similar to molybdopterin oxidoreductase [*Alkalilimnicola ehrlichei* MLHE-1, E value 5.6]	2.7	1.1	+	
yqjZ	unknown; similar to antibiotic biosynthesis monooxygenase [*Pseudomonas fluorescens* PfO-1, E value 3e-31]	5.9	1.5	+	MMC, UV
yqjY	unknown; similar to acetyltransferase, GNAT family [*B. anthracis* str. Ames, E value 5e-40]	2.8	1.1	+	MMC, UV
yqjX	unknown; similar to similar to YolD homolog [*B.* sp. NRRL B-14911, E value 1e-04]	3.8	1.5	+	MMC, SA, UV
yqjW	unknown; similar to DNA polymerase IV 2 (Pol IV 2), ATP/GTP-binding protein (ImpB/MucB/SamB family) [*B. halodurans* C-125, E value 1e-142]; DNA-damage repair protein [*Oceanobacillus iheyensis* HTE831, E value 1e-81]	7.4	1.4	+	MMC, SA, UV
yqjI	unknown; similar to 6-phosphogluconate dehydrogenase (pentose phosphate) [*B. subtilis* subsp. *subtilis* str. 168, E value 0.0]	5.1	1.8		
yqjH	unknown; similar to DNA polymerase IV [*B.* sp. NRRL B-14911, E value 1e-130]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 7e-83]	8.5	2.1		
yqzJ	unknown; similar to hypothetical protein BSU23880 [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-48]	4.0	3.5		
yqiZ	unknown; similar to amino acid ABC transporter, ATP-binding protein [*B. cereus* ATCC 10987, E value 4e-94]	4.1	2.4		HS
comEB	late competence operon required for DNA binding and uptake (non-essential gene for competence; similar to several dCMP deaminases)	3.0	1.7		PS
arsB	extrusion of arsenite / putative heavy metal membrane efflux protein	2.5	2.0		
phrE	phosphatase (RapE) regulator	4.0	1.9		
yrdQ	unknown; similar to transcriptional regulator, LysR family [*B. anthracis* str. Sterne, E value 1e-124]	3.2	2.8		
trkA	potassium uptake	2.6	2.4		
aadK	aminoglycoside 6-adenylyltransferase	5.0	1.7		
udk	uridine kinase	2.6	1.7		
yrrK	unknown; similar to Holliday junction resolvase-like protein [*B. subtilis* subsp. *subtilis* str. 168, E value 8e-72]	11.7	1.7		
yrvE	unknown; similar to single-strand DNA-specific exonuclease (RecJ) [*B. licheniformis* ATCC 14580, E value 0.0]	8.4	1.4		GS, HS
ruvB	Holliday junction DNA helicase	6.8	1.8	+	MMC, UV
radC	probable DNA repair protein	7.0	2.6		
ytkD	unknown; similar to phosphohydrolase (MutT/nudix family protein) [*B. cereus* ATCC 14579, E value 1e-51]; 7,8-dihydro-8-oxoguanine-triphosphatase [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-51]	9.4	3.7		
yukA	unknown; similar to DNA segregation ATPase [*B. clausii* KSM-K16, E value 0.0]	2.9	1.4		AG
yuiA	unknown; similar to DnaJ-class molecular chaperone with C-terminal Zn finger domain [*Rubrivivax gelatinosus* PM1, E value 0.068]; phosphoesterase (RecJ-like) [uncultured methanogenic archaeon RC-I, E value 0.20]	5.1	1.8		PS
pucB	xanthine dehydrogenase (purine degradation)	3.8	2.9		
mrgA	metalloregulation DNA-binding stress protein	8.1	3.9	+	PS, SA^R
gerAB	germination response to L-alanine	2.8	2.3		
bdbB	thiol-disulfide oxidoreductase	4.1	2.6		

uvrA	excinuclease ABC (subunit A)	6.8	1.9	+	MMC, PS, UV
uvrB	excinuclease ABC (subunit B)	4.7	1.1	+	MMC, PS, UV
tagC	possibly involved in polyglycerol phosphate teichoic acid biosynthesis (DinR-binding site; DNA-damage inducible; *dinC*)	22.8	1.8	+	MMC, PS, UV
ywqL	unknown; similar to deoxyinosine 3'endonuclease [*C. beijerincki* NCIMB 8052, E value 1e-80]	5.7	1.3		
ywqA	unknown; similar to helicase YwqA [*B. licheniformis* ATCC 14580, E value 0.0]	7.0	1.9		
ywoG	unknown; similar to antibiotic resistance protein (antibiotic efflux protein) [*Oceanobacillus iheyensis* HTE831, E value 4e-85]	2.5	1.7		
atpB	ATP synthase (subunit a)	3.9	2.7		
pyrG	CTP synthetase	2.3	1.4		
ywjD	unknown; similar to UV-endonuclease (UvsE/Uve1/UvdE Family) [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-109]	15.1	6.2		
ywcH	Unknown; similar to oxygenase [*B. licheniformis* ATCC 14580, E value 1e-118]	9.0	4.7		
exoA	multifunctional DNA-repair enzyme	4.4	3.1		
ssb	single-strand DNA-binding protein	16.5	4.7		MMC, UV
yyaF	Unknown; similar to GTP-dependent nucleic acid-binding protein EngD [*B. licheniformis* ATCC 14580, E value 1e-177]	3.4	2.4		

[a] For details on the data presentation, see Table 3-8, footnotes [a] through [e].

To analyze the function of the up-regulated transcripts after 1 h germination, overrepresented groups of functional related genes (gene ontologies groups, GO) were identified by using a comprehensive bibliographical database search approach (chapter 2.4, 2.4.4 - 2.4.6). In Table 3-14 seven gene ontology groups are listed and the most prominent functional categories in relation to their gene expression were discussed.

Table 3-14 Highly UV-A induced genes and their functional affiliation in 7 gene ontology groups[a]

GO 1. DNA repair and modification/restriction

wild-type: *adaB, alkA, dinB, exoA, lexA, mutS, radC, tagC (dinC), uvrA, uvrB, uvrX, yacK (disA), ydiO, ydiP, yhaO, yhaZ, yirY, yisB, yjcD, ykoQ, ymaB, yobH, yqjH, yqjW, ytkD, ywjD*

recA: *adaB, alkA, exoA, mutS, radC, uvrX, ykoQ, ymaB, ytkD, ywjD*

GO 2. DNA recombination

wild-type: *recA, recF, recU, ruvB, yefB, yefC, yneB, yocI, yokA, yorS, yrrK, yrvE, ywqA*

recA: -

GO 3. DNA replication

wild-type: *dnaA, dnaN, ligB, pcrA, polC, ssb, yomH•, yorI, yorL*

recA: *ssb*

GO 4. DNA packaging and segregation

wild-type: *hbs, topB, yddE, yolD•, yopB•, yopW•, yoqP•, yorA, yozL•, yqjX, yukA*

recA: *yddE, yorA*

GO 5. Nucleotides, nucleobases, nucleotide binding and nucleic acid metabolism

wild-type: *adk, gmk, ndk, pbuG, pbuX, pucB, purR, pyrG, pyrR, tmk, udk, ykoW, yomO•, yonF, yonV, yorG, ywqL, yyaF*

recA: *ndk, pbuG, pucB*

GO 6. Adaptation, detoxification and response to stress (UV-A stimulus)

wild-type: *aadK, arsB, bdbB, mrgA, ykuU, ymaD, yonG•, yppQ, yqjZ, yqkB•, yuiA•, ywcH*

recA: *bdbB, mrgA, ymaD, yppQ, ywcH*

GO 7. Other functions

GO 7.1 Germination and cell envelope

wild-type: *gerAB, murG, yocA, yocH*

recA: -

GO 7.2 Bioenergetics

wild-type: *atpB, pksB*

recA: *atpB*

GO 7.3 Other functions, or/and unknown and hypothetical proteins

wild-type: *comEB, cotE, cotJC, mraY, phrE, phrK, pnpA, prsA, sacV, sigW, trkA, uppS, yaaC, ybaL, ybaR, ydiM, yhaU, yhaX, yjcG, ylaC, yobE, yocJ, yomE, yomR, yomS, yomW, yomX, yopA, yopT, yopX•, yoqA•, yqiZ, yqjI, yqjY, yqzJ, yrdQ, ywoG*

recA: *cotE, phrK, yqzJ, yrdQ*

• Gene with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). For details on the gene function, see Table 3-13. [a] For details on the data presentation, see Table 3-9.

Among these highly up-regulated transcripts many genes have described functions that may be directly involved in DNA repair, recombination and replication processes (Table 3-14, gene ontology groups GO 1.-3.) and genes involved in nucleotide stability and integrity (GO 5.).

In the wild-type strain, typical UV induced DNA repair genes, e.g. general DNA damage-inducible genes *dinB* and former *dinC* (*tagC*), the NER (nucleotide excision repair) exonucleases *uvrA* and *uvrB*, the probable DNA repair protein *radC* and *recA*, the multifunctional protein involved in the homologous recombination (and recombination-mediated repair) and the single-strand DNA-binding encoding gene *ssb* showed significantly increased fluorescent values, as can be seen in Table 3-13. The multifunctional DNA-repair enzyme ExoA, an AP endonuclease and 3'-5' exonuclease, was up-regulated. In addition to known DNA repair enzyme encoding genes four "*y*"-genes, predicted DNA repair transcripts were up-regulated: *yhaO* (a *B. thuringiensis* str. Al Hakam-like DNA repair exonuclease), *yhaZ* (a probable DNA alkylation repair enzyme, similar to *Lactobacillus sakei* subsp. *sakei* 23K), *yirY* (a putitative DNA repair exonuclease or specific DNA repair related ATPase) and *ykoQ* (a DNA repair exonuclease and/or phosphoesterase), which was previous mentioned in its UV-C response. Two putitative UV-A responsive transcripts, *yjcD* (an UvrD-like DNA helicase) and *ywjD* (an UV-endonuclease), were also found to be activated and involved in UV-A damage repair, similar to their induction after UV-C irradiation.

Nine different DNA polymerases, five DNA polymerases type III, DNA polymerases directly involved in the DNA replication and elongation: *dnaN, polC, yorS, yorL,* and *yomH*; and four DNA polymerase type IV (DinB; damage-inducible): *uvrX, yobH, yqjH* and *yqjW* were significantly induced after 60 min germination (Table 3-13).

Two mutT/nudix family protein encoding genes, *ymaB* and *ytkD* (similar to *B. cereus* ATCC 14579) were highly up-regulated. Previously, *yktD* was found to be involved in the cell resistance to oxidative stress, as a potential 8-oxo-dGTPase (Castellanos-Juárez et al., 2006). In addition to these putitative antimutator proteins, *mutS* a DNA mismatch recognizing protein was also activated in the irradiated spores during spore germination to ensure DNA nucleotide mismatch removal.

Eleven, significantly up-regulated, genes can be directly assigned to UV-A stress response e.g. removal of UV-A induced reactive oxygen species formed damage and detoxification processes, especially *ywcH* (a *B. licheniformis* ATCC 14580-like oxygenase), *ymaD* (a probable hydroperoxide resistance protein, similar to the OsmC-protein of *Legionella pneumophila* subsp. *pneumophila* str. Philadelphia

1), *yppQ* (a predicted sulfoxide reductase, similar to *B. subtilis* subsp. *subtilis* str. 168) and *ykuU* (a *B. licheniformis* ATCC 14580-like alkyl hydroperoxide reductase) showed high levels of transcriptional activation (Table 3-14, GO 2.).

RecA, *recF* and *recU* involved in SOS-induced DNA repair processes (Fernandez et al., 1998) were also significantly induced by UV-A radiation. Further on, nine "*y*"-genes which are expected to be involved in the homologous recombination (and recombination-mediated repair) were significantly induced; six recombinase/resolvase encoding genes: *yefB* (similar to *Geobacillus kaustophilus* HTA426), *yefC* (similar to *B. cereus* G9241), *yneB* (a putative N-terminal resolvase/recombinase), *yrrK* (Holliday junction resolvase-like protein) and *yokA* (a predicted N-terminal resolvase/recombinase, similar to *B. weihenstephanensis* KBAB4); two helicases: *yocI* (ATP-depending DNA helicase) and *ywqA* (a *B. licheniformis* ATCC 14580-like encoding helicase) and the *recJ*-like single-stranded-DNA specific exonuclease *yrvE* (Table 3-14, GO 2.).

Eighteen genes were found to be induced and involved in gene ontology group "nucleotides, nucleobases, nucleotide binding and nucleic acid metabolism", especially the nucleotide kinases and genes involved in the purine and pyrimidine metabolism are likely to be also implicated in the new synthesis of DNA nucleotides, essential for the replacement of damage-modified DNA bases (Table 3-14, GO 5.).

A deletion in the major recombinogenic protein *RecA* led to an about 2-fold decrease in the DNA PP removal after UV-A irradiation during spore germination (Table 3-12) and to a less specific transcriptional response (as can seen in Table 3-13). The five highest up-regulated genes in the *recA*-deficient strain were *ywjD* (a putative UV-endonuclease), *ymaB* (a mutT-like protein), *ywcH* (a predicted oxygenase), *ssb* (a single-strand DNA-binding protein) and *ykoQ* (a probable DNA repair exonuclease).

To verify the transcriptional response of the induced, putative DNA exonuclease/phoshoesterase (highly similar to *B. cereus* E33L) *ykoQ* (7.3 after UV-C (Table 5-8), 8.0 after UV-A irradiation (Table 3-13), 14.4 after X-ray- and 9.9 after Fe 500-irradiation (Table 3-20) and 60 min germination) was

selected for RT-PCR experiments (2.4.7). Fig. 3-15 shows the DNA bands of the amplified cDNA after RT-PCR (as described in 2.4.7), the rows loaded with: 1 non-UV-A irradiated (control), 2 UV-C irradiated, 3 UV-A irradiated, 4 X-rays irradiated 5 accelerated Fe-ions (500 MeV/n) irradiated and M standard DNA marker (Hyper Ladder IV, Bioline GmbH, Luckenwalde, Germany). The amplified cDNA band for the estimated RT-PCR product was in the expected product size (234 bp).

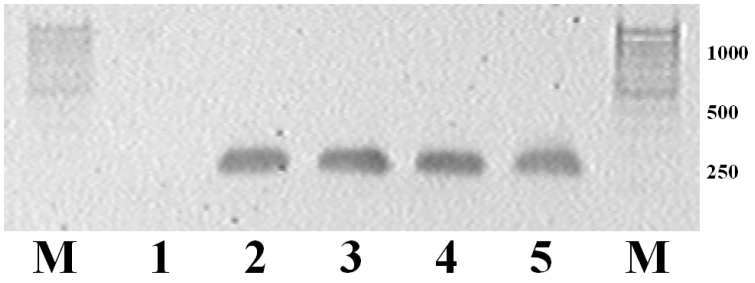


Fig. 3-15 RT-PCR analysis of UV and ionizing radiation exposed (2, 3, 4, 5) and non-irradiated (1) wild-type *B. subtilis* 168 *ykoQ* - transcript after 60 min germination.

Similar cDNA band pattern were obtained for *ymaD* (a predicted hydroperoxide resistance protein), *yobH* and *uvrX* (two predicted UV damage-repair error-prone polymerases) from RNA samples isolated out of UV-A irradiated and non-irradiated 60 min-germinating wild-type *B. subtilis* 168 spores (for functional characterization and sequence homologies see Tables 3-13 and 3-14).

3.4 Effects of ionizing radiation on dormant *B. subtilis* spores

Application of ionizing radiation to sterilize food, medical devices, biohazardous material (i.e. mail potentially contaminated with *B. anthracis* spores; according to Niebuhr and Dickson, 2003), the isolation of microorganisms from habitats with high level of surface radiation as well as the astrobiological interest in the response of bacteria and archaea to galactic cosmic rays have led to a number of studies on the radio-resistance of microorganisms. Especially the radiation environments on Earth, Mars, in low-Earth orbit and in outer space are typified by a wide variety of particles covering an extended range of energies. Galactic cosmic rays (GCR) are charged particles that originate from sources beyond our solar system. The distribution of GCRs is assumed to be isotropic throughout the interstellar space. The spectrum of the GCRs consists of 98 % protons and heavier ions (baryon component) and 2 % electrons and positrons (lepton component). The baryon component is composed of 87 % protons, 12 % helium ions (alpha particles) and the remaining 1 % heavy ions of charge 3 (Li)

- 92 (U), also called particles of High charge Z and energy E (HZE particles; Horneck, 1994). These HZE particles, especially iron-ions, are of high-LET and are highly penetrating through matter, giving them a large potential for radiobiological damage. LET, linear energy transfer is a measure of the energy transferred to material as an ionizing particle travels through it. It is typically used to quantify the effects of ionizing radiation on biological specimens.

3.4.1 Survival of *B. subtilis* spores after exposure to ionizing radiation

Dose-effect curves of inactivation of spores by X-rays (150 keV) as well as by various accelerated heavy ions of different charge and energy (Table 2-4, 2.2.4.2, 2.5, 2.5.1) are given in Fig. 3-16. Spores of the wild-type (*B. subtilis* 168), of the *recA* or major SASPs-deficient (*sspA sspB*) mutants were studied. Table 3-15 summarizes the curve characteristics. All survival curves of *recA* spores and of *sspA sspB* spores are purely exponential (within the experimental error). The wild-type spores show slightly shouldered survival curves for X-rays or accelerated He ions, indicated by an extrapolation number above 1.0 (Table 3-15).

Irradiation with C, Si or Fe ions led to exponential survival curves also for the wild-type spores. Comparison of the D_0-values (Table 3-15) shows that spores defective in the major small acid-soluble spore proteins were about 3.5-times more sensitive to ionizing radiation than the wild-type SASPs spores, and the *recA*-deficient spores were about 5-times more affected. The extremely high resistance of wild-type *B. subtilis* spores to ionizing radiation becomes visible when comparing their D_0 values (D_0 = 555 Gy for X-rays) with those of their vegetative cells (D_0 = 33 Gy for X-rays: Horneck et al., 1989; Micke et al., 1994). One explanation for the high radiation-resistance of bacterial spores might be their reduced water content of the spore core (less than 30 %; according to Algie, 1984), which may hinder the formation of hydroxyl radicals by ionizing radiation (reviewed in Nicholson et al., 2000).

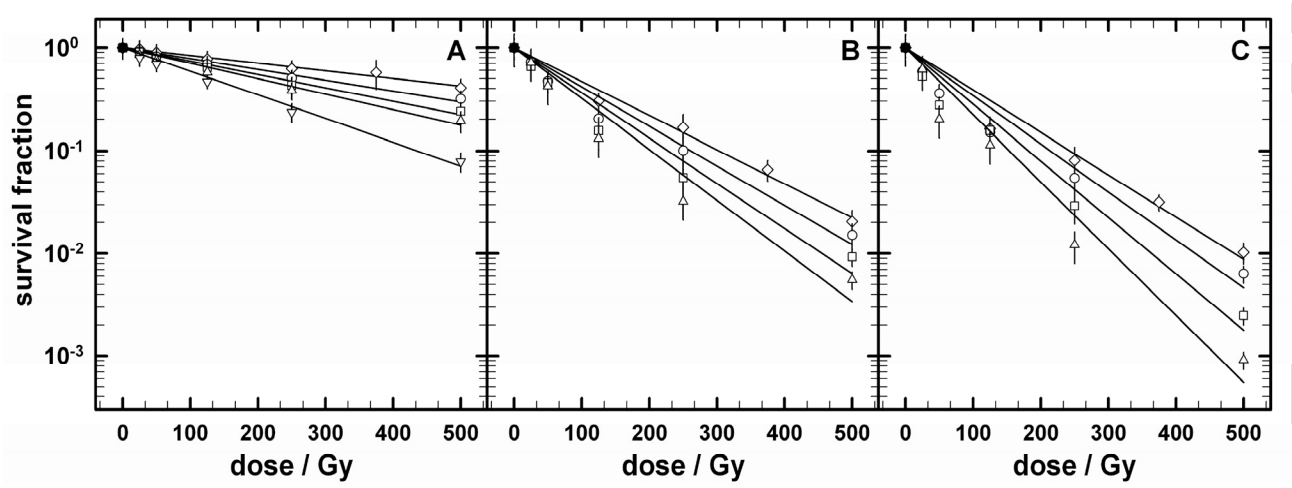


Fig. 3-16 Dose-survival curve of *B. subtilis* wild-type (**A**), *recA* (**B**) and *sspA sspB* (**C**) spores after exposure to X-rays (150 keV; open diamonds) or to accelerated heavy ions: He (150 MeV/n, 2.2 keV/µm; open circles), C (400 MeV/n, 12 keV/µm; open squares), Si (490 MeV/n, 50 keV/µm; open triangles up), Fe (500 MeV/n, 200 keV/µm; open triangles down). Data are averages ± standard deviation ($n = 4$).

Table 3-15 Survival curve characteristics after exposure to ionizing radiation (X-rays and accelerated heavy ions)

X-rays (150 keV/19 mA)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	1039.8 ± 146.7[a]	578.1 ± 62.9[a]	43.5 ± 8.7	(1.70 ± 0.27)×10^{-3}
sspA sspB	301.6 ± 58.9	125.7 ± 32.1	n.d.	(7.44 ± 1.57)×10^{-3}
recA	203.4 ± 32.7	78.9 ± 27.6	n.d.	(8.65 ± 1.98)×10^{-3}

X-rays (150 keV/19 mA)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.21 ± 0.09	1	0.980	
sspA sspB	0.87 ± 0.11	0.29 ± 0.07	0.963	0.017
recA	0.73 ± 0.07	0.19 ± 0.09	0.975	0.019

He 150 MeV/n (LET 2.2 keV/µm)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	857.3 ± 95.2[a]	408.6 ± 48.3	13.5 ± 4.0	(2.27 ± 0.26)×10^{-3}
sspA sspB	235.9 ± 41.4	86.2 ± 20.7	n.d.	(8.09 ± 0.79)×10^{-3}
recA	187.9 ± 24.6	59.3 ± 16.7	n.d.	(9.67 ± 1.41)×10^{-3}

He 150 MeV/n (LET 2.2 keV/µm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.18 ± 0.10	1	0.989	
sspA sspB	0.79 ± 0.15	0.27 ± 0.08	0.982	0.009
recA	0.69 ± 0.08	0.21 ± 0.11	0.979	0.007

C 400 MeV/n (LET 12 keV/µm)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	723.1 ± 67.3[a]	321.7 ± 48.3	7.2 ± 2.1	$(2.86 \pm 0.33)\times10^{-3}$
sspA sspB	198.7 ± 38.3	70.0 ± 19.6	n.d.	$(9.19 \pm 1.02)\times10^{-3}$
recA	158.4 ± 19.1	49.1 ± 8.1	n.d.	$(1.16 \pm 0.29)\times10^{-2}$

C 400 MeV/n (LET 12 keV/µm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.07 ± 0.07	1	0.986	
sspA sspB	0.73 ± 0.12	0.26 ± 0.09	0.974	0.011
recA	0.65 ± 0.09	0.20 ± 0.05	0.986	0.008

Si 490 MeV/n (LET 50 keV/µm)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	649.3 ± 55.2[a]	276.3 ± 22.6	n.d.	$(3.21 \pm 0.28)\times10^{-3}$
sspA sspB	178.4 ± 21.8	62.7 ± 12.7	n.d.	$(1.04 \pm 0.17)\times10^{-2}$
recA	122.0 ± 14.1	38.2 ± 5.8	n.d.	$(1.39 \pm 0.11)\times10^{-2}$

Si 490 MeV/n (LET 50 keV/µm)	n	RF	R^2	*P*
B. subtilis (wild-type)	0.97 ± 0.06	1	0.988	
sspA sspB	0.68 ± 0.11	0.28 ± 0.05	0.960	0.017
recA	0.59 ± 0.07	0.18 ± 0.04	0.974	0.015

Fe 500 MeV/n (LET 200 keV/µm)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	436.6 ± 35.0	176.5 ± 22.3	n.d.	$(4.99 \pm 0.35)\times10^{-3}$
sspA sspB	n.t.	n.t.	n.t.	n.t.
recA	n.t.	n.t.	n.t.	n.t.

Fe 500 MeV/n (LET 200 keV/µm)	n	RF	R^2	*P*
B. subtilis (wild-type)	0.90 ± 0.07	1	0.993	
sspA sspB	n.t.	n.t.	n.t.	n.t.
recA	n.t.	n.t.	n.t.	n.t.

n.d. not determinable; n.t. not tested. [a]Value obtained by extrapolation. The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). *P* = statistical significance of difference of data of respective strain compared to *B. subtilis* wild-type strain 168; $P \leq 0.05$ = significant difference. Data are expressed as averages and standard deviations ($n = 4$).

It is interesting to note that SASPs deficient spores exhibited a significant inactivation by ionizing radiation (3.5-times more that the wild-type spores); this increased radiation sensitivity is probably based on the absence of DNA-SASP binding. Previous studies have shown a higher frequency of single-strand breaks in the DNA of $\alpha^-\beta^-$ spores killed by oxidizing agents than in the DNA of wild-type spores treated in the same way (Setlow, 1995). This leads to the assumption that the SASPs play a role in the resistance of spores to ionizing radiation, similar as in the case of UV radiation. As a major structural component SASPs may ensure nucleoid integrity and/or serve as possible scavengers for ROS. Biochemical studies on SSB and DSB induced in the DNA of $\alpha^-\beta^-$ irradiated spores and their repair during germination support the assumption that SASPs have an essential role in the spore resistance to ionizing radiation (Setlow, 1995; 2006; Nicholson et al., 2000). However, the mechanism involved is still not well understood.

The high radiation sensitivity of spores of the *recA* strain (about 5 times more sensitive that wild-type spores) confirms earlier observations by Micke et al. (1994) and Baltschukat et al. (1986) who monitored the repair DSBs in heavy ion irradiated vegetative cells by using a *B. subtilis* strain carrying a triple mutation in *recA*, *uvrB* and *splB* (strain TKJ8431, for details see Table 2-1, 2.1.2). They found out that a *recA* deficiency led to a dramatic decrease in DSB repair efficiency of vegetative cells. The lack in recombination-mediated repair cannot be the reason, because this mechanism (homologous recombination) requires at least 2 chromosomes per cell and spores are monogenomic; homologous recombination can not operate during spore germination of wild-type cells. These observations indicate that genetic (homologous) recombination (and recombination-mediated repair) may probably not be a main repair pathway for spores irradiated by ionizing radiation. Other repair pathways e.g. NHEJ (non-

homologous end-joining) have to be considered for accomplishing DNA strand break repair in germinating spores. On the other hand, *recA*-deficiency of the spores led to aggrevated inactivation by ionizing radiation. This may be based on another function of RecA, namely the unique role of RecA in genome replication during mid germination and outgrowth (Sciochetti et al., 1999; 2001).

3.4.2 Mutation induction in spores after exposure to ionizing radiation

For studying the induction of mutations caused by ionizing radiation spores were selected for the change in antibiotic resistance (2.3.3). The antibiotic rifamicin (Rif) was chosen, because of its role as one of the most common used inhibitors of the prokaryotic transcription initiation (Wehrli et al. 1968). Rif has long been used to study the transcription in bacteria. It has also been used as a highly effective clinical drug, particularly in the treatment of tuberculosis. Mycobacterial resistance to Rif can arise from mutations in the *rpoB* gene encoding the β subunit of RNA polymerase; the vast majority (96 %) of these mutations occurs within a short 69-bp stretch of *rpoB* (Nicholson and Maughan, 2002) which corresponds to Rif resistance (Rif^r) cluster I (Fig. 3-17) in the well-characterized *Escherichia coli* K-12 (Jin and Gross, 1988).

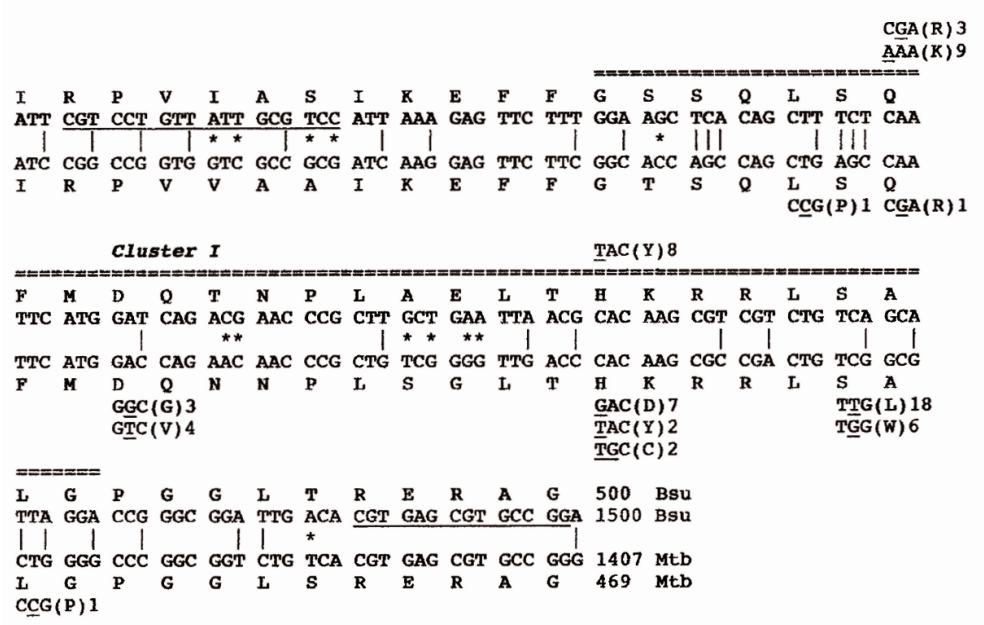


Fig. 3-17 Comparison of the Rif-resistance regions (nucleotide and amino acid sequences) of *rpoB* in *B. subtilis* (Bsu; top two lines, Nicholson and Maughan, 2002) and *M. tuberculosis* (Mtb; bottom two lines). The double dashed line indicates the region corresponding to Rif^r cluster I in the *E. coli rpoB* gene (Jin and Gross 1988).

Nucleotide mismatches which are silent (vertical lines) or lead to amino acid differences (asterisks) are denoted between the sequences. Mutations leading to Rif^r in *B. subtilis* (Boor et al. 1995) and *M. tuberculosis* (Gracia et al., 2001) are denoted above or below the wild-type sequences. The numbers beside each mutation refer to the number of independent isolations of that mutation (Nicholson and Maughan, 2002).

In *B. subtilis*, a single Rif^r mutation, called *rfm2103*, was found to reside in *rpoB* (Boor et al., 1995), and more recently a larger collection of Rif^r mutations, spontaneous and generated by ethyl methanesulfonate or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine mutagenes, was characterized for *B. subtilis* 168 (Ingham and Furneaux, 2000). All mutations isolated so far from *B. subtilis* are single nucleotide substitutions resulting in specific amino acid changes and are located within the portion of *rpoB* which corresponds to cluster I at only two positions, Q469R or Q469K (Q glutamine to R arginine or to K lysine, respectively) or at H482Y (H histidine to Y tyrosine) (Nicholson and Maughan, 2002). While in clinical isolates of *Mycobacterium tuberculosis* several different mutations, including single C-to-T transitions (absent in *B. subtilis*), in cluster I of *rpoB* were observed, the most abundant Rif^r mutation occured at amino acids (using *B. subtilis* 168 *rpoB* coordinates) S487L (S serine to L leucine) and S487W (S serine to W tryptophan) (Kim et al., 2001).
Spores *B. subtilis* 168 exhibit a spectrum of spontaneous Rif^r mutation distinct from that of vegetative cells. Mutations such as Q469K, Q469R and H482Y as well as new mutations of *rpoB*, H482R (H histidine to R arginine) were determined from vegetative cells and A478N (A alanine to N asparagine) and S487L for spores, respectively. These genetic characterizations and physiological observations led to the calculation of the spontaneous mutation rate to Rif^r as 1×10^{-8} for vegetative cells and 5×10^{-9} for spores (Nicholson and Maughan, 2002).

Mutation to rifampicin resistance was assayed on NB medium containing 50 µg of rifamicin per ml (as described in 2.3.3). In all tested strains, identical mutation induction rates were obtained for X-rays as well as for accelerated Si-ions, the only ion applied to all three strains (Table 3-16). With increasing LET of the applied ions, the mutation induction rate increased as well (data available for wild-type spores only) (Fig. 3-18 and Table 3-16). Mutation to rifampicin resistance was assayed on NB medium containing 50 µg of rifamicin per ml (as described in 2.3.3). The mutation induction as function of the

applied dose of ionizing radiation is shown in Fig. 3-18. The non-treated controls showed a spontaneous mutation rate in the range of 2.2-4.6×10^{-9} for the three test strains, in agreement with data from Nicholson and Maughan (2002). Within experimental errors, exponential mutation induction curves were obtained. With increasing LET of the applied ions, the mutation induction rate increased (data for all ions available for wild-type spores only) (Fig. 3-18 and Table 3-15). These findings confirm previous studies on mutation induction in *B. subtilis* spores - in that case to resistance to sodium azide - by accelerated heavy ions, that showed an increasing mutation induction rate with rising LET of the particle radiation (Baltschukat et al., 1986). For X-rays or for accelerated Si-ions - the only ion applied to all three strains - identical mutation induction rates were obtained for all three tested strains (Table 3-16). After irradiation at 0.5 kGy (the highest dose applied in this work) the mutation rates increased by about one order of magnitude for X-rays and by about two orders of magnitude for Si ions.

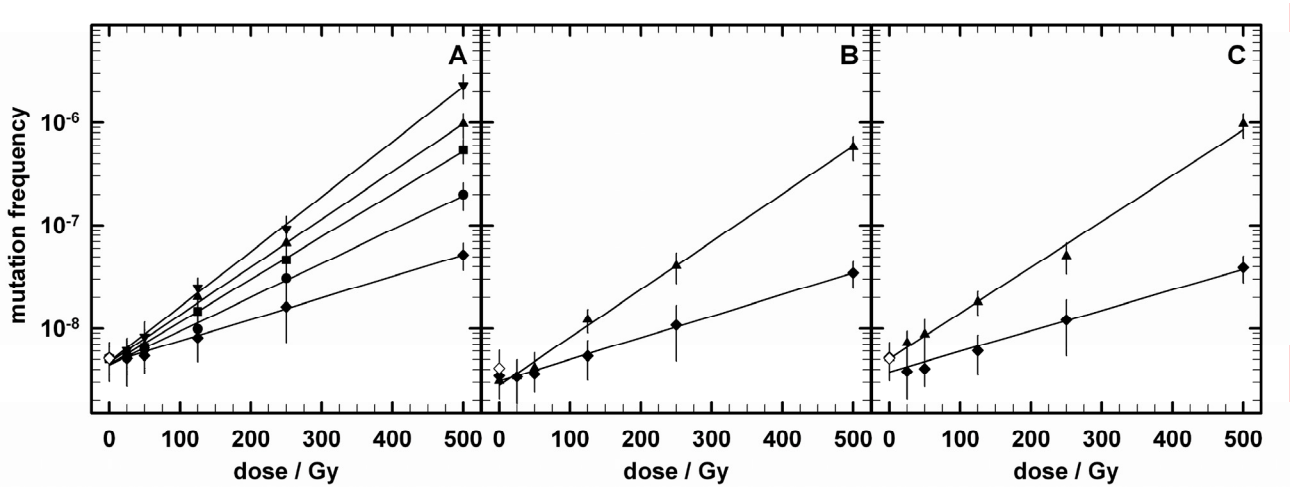


Fig. 3-18 Dose-effect curves for mutation induction to Rif^r in *B. subtilis* 168 wild-type (**A**), *recA* (**B**) and *sspA sspB* (**C**) spores exposed to X-rays (150 keV; solid diamonds) or accelerated heavy ions: He (150 MeV/n, 2.2 keV/µm; solid circles), C (400 MeV/n, 12 keV/µm; solid squares), Si (490 MeV/n, 50 keV/µm; solid triangles up) and Fe (500 MeV/n, 200 keV/µm; solid triangles down); and spontaneous mutation rate (open circles). Data are reported as averages and standard deviations (n = 3). Mutation induction frequency was calculated using (*Eq. 3*) as described in 2.5.

Table 3-16 Curve characteristic for Rif^r induction by ionizing radiation

		Mutation induction rate[a]	
Ionizing radiation	*B. subtilis* (wild-type)	*recA*	*sspA sspB*
X-rays (150 keV)	$(2.08 \pm 0.35)\times10^{-3}$	$(2.12 \pm 0.43)\times10^{-3}$	$(1.98 \pm 0.52)\times10^{-3}$
He 150 MeV/n	$(3.29 \pm 0.54)\times10^{-3}$	n.t.	n.t.
C 400 MeV/n	$(4.15 \pm 0.68)\times10^{-3}$	n.t.	n.t.
Si 490 MeV/n	$(4.65 \pm 0.58)\times10^{-3}$	$(4.48 \pm 0.82)\times10^{-3}$	$(4.30 \pm 1.06)\times10^{-3}$
Fe 500 MeV/n	$(5.34 \pm 0.73)\times10^{-3}$	n.t.	n.t.

n.t. not tested. Data are averages and standard deviations (n = 3). [a]Mutation induction rate was obtained from the slope of the exponential mutation induction curve (Fig. 3-18A- C).

Thirty different Rif^r mutants induced in *B. subtilis* 168 spores either by irradiation with 0.25 kGy or 0.5 kGy X-rays or accelerated heavy ions (He 150, C 400, Si 490 and Fe 500) or by exposure to high vacuum (15, 30 and 450 d) (see chapter 3.5) were isolated. (Mutations were pooled for this purpose; however, it was always possible to trace back the history of each individual induced mutation.) They arose from separate selections on Rif-containing medium plates, were streak purified, PCR amplified and sequenced as described previously in 2.3.3.

As a control, chromosomal template DNA prepared in parallel from a colony of *B. subtilis* strain 168 grown overnight on NB agar plates was amplified and sequenced; its *rpoB* sequence was identical to previously published ones (Boor et al., 1995; Ingham and Furneaux, 2000).

Sequence analysis of the cluster I region of *rpoB* in the 30 Rif^r isolates showed a clear difference in the spectrum of induced (by radiation or high vacuum) Rif^r mutations (Fig. 3-19) compared to the spontanous Rif-resistance spectrum, the latter taken from Nicholson and Maughan (2002) (Fig. 3-17).

463	464	465	466	467	468	469	470	471	472	473	474	475
G	S	S	Q	L	S	Q	F	M	D	Q	T	N
GGA	AGC	TCA	CAG	CTT	TCT	CAA	TTC	ATG	GAT	CAG	ACG	AAC
				C**C**T		C**T**A						
				(P)		(L)						

476	477	478	479	480	481	482	483	484	485	486	487	488	489	490
P	L	A	E	L	T	H	K	R	R	L	S	A	L	G
CCG	CTT	GCT	GAA	TTA	ACG	CAC	AAG	CGT	CGT	CTG	TCA	GCA	TTA	GGA
		G**T**T				**T**AC		C**C**T			T**T**A	**C**CA		
		(V)				(Y)		(P)			(L)	(P)		
						C**C**C								
						(P)								

Fig. 3-19 Spectrum of Rif^r mutations in *B. subtilis* 168 *rpoB* gene that were induced in spores exposed to ionizing radiation or high vacuum. The wild-type *rpoB* nucleotide and amino acid sequence are shown for cluster I (from positions G463 to G490). Positions of nucleotide changes leading to the indicated amino acid substitutions in irradiated spores are denoted below the wild-type sequence and the exact nucleotide change is underlined. Amino acid changes resulting from each mutation are in parentheses.

Out of thirty Rif^r mutations sequenced that were induced in spores of *B. subtilis* 168 exposed either to ionizing radiation or high vacuum all but two were based on changes in the cluster I of the *rpoB* gene and were caused either by a single transversion or a single transition (Table 3-17). The major class of Rif^r mutations observed after exposure of spores to ionizing radiation or high vacuum consisted of a unique A-to-T transversion resulting in Q469L (7), an A-to-C transversion in H482P (5), a C-to-T transition in A478V (4) or a C-to-T transition in S487L (4). Q469R, H482P, H482Y and S487L mutations have been observed previously in *B. subtilis rpoB* (Maughan et al., 2002), but the L467P, A478V, R484P and A488P mutations are new mutations uncovered in this work. A comprehensive comparison of all *rpoB* mutations isolated in this work after induction in *B. subtilis* spores by ionizing radiation or vacuum is presented in Table 3-17 and Fig. 3-20 the frequency distribution of the induced mutations are shown. The already known mutations Q469L and H482P in cluster I of the *B. subtilis* 168 *rpoB* gene were the most frequently observed changes in this work as well as in earlier mutation studies (Nicholson and Maughan, 2002). Those two predominant mutations were found after irradiation of spores with different qualities of ionizing radiation, after treatment with high vacuum, after stress

impact of extreme dryness (Munakata et al., 2004) as well as a major spontaneous Rif^r mutation (Nicholson and Maughan, 2002). Such susceptible regions of the prokaryotic genome that are prone to mutational alteration are known under the term "hot spots". Therefore, those two sites are termed "mutational hot spots". For comparison, another mutational hot spot has been reported for mutations in *gyrA*, the DNA gyrase subunit A encoding gene and key factor in resistance to nalidixic acid. After irradiation of spores with UV, vacuum-UV, γ-radiation or X-rays, a unique tandem-base change mutation in one particular allele of *gyrA* (A12 5'-CA to 5'-TT) was predominantly induced (Munakata et al., 1997). Of course, in all these studies one has to bear in mind that the detection of mutational specificities depends also on several experimental parameters, such as the size of the region concerned, the readiness with which the mutation can be detected, and the possibility that selection against mutants at that point is less than that against mutants elsewhere.

Table 3-17 Summary of mutational changes leading to rifamipcin resistance (Rif^r) in the *rpoB* gene of *B. subtilis* 168 spores exposed to ionizing radiation or high vacuum

CP[a]	Codon change (Transversion)	Codon change (Transition)	aa change[b]	Rif^r mutations isolated in *B. subtilis* 168 spores[c/d]				
				number	(%)	0.25 kGy	0.5 kGy	vacuum / d
467		CTT to C**C**T	L to P	1	3.33		Fe	
469	CAA to C**T**A		Q to L	7	23.33	Fe, Si	C, Fe, He,	30, 450
478		GCT to G**T**T	A to V	4	13.33	Si	He, X-rays	450
482		CAC to **T**AC	H to Y	2	6.67	C	X-rays	
482	CAC to C**C**C		H to P	5	16.67	Si, X-rays	C	30, 450
484	CGT to C**C**T		R to P	3	10.00	He	Fe, He	
487		TCA to T**T**A	S to L	4	13.33	Si	C	15, 450
488	GCA to **C**CA		A to P	2	6.67		Si	15
504		CGT to C**A**T	R to H	1	3.33			450
507		CAC to C**G**C	H to R	1	3.33		Fe	

[a]Codon positions (CP) using *B. subtilis* 168 coordinates. [b]aa change = amino acid change. [c]30 Rif^r mutations were obtained and sequenced from ionizing radiation and high vacuum exposed *B. subtilis* spores after cultivation (germination and outgrowth) of their vegetative cells. [d]Detailed on the exposure to ionizing radiation and high vacuum are described in detail in 2.2.4.1, 2.2.4.2 and 2.2.5.

Additionally to the found and previously characterized *rpoB* changes, four new Rif^r mutations in cluster I arising from irradiated spores were detected: L467P, A478V, R484P and A488P, as well as two changes located in codon R504H and H507R, i.e. between cluster I and II (Campbell et al., 2001) (Fig. 3-20).

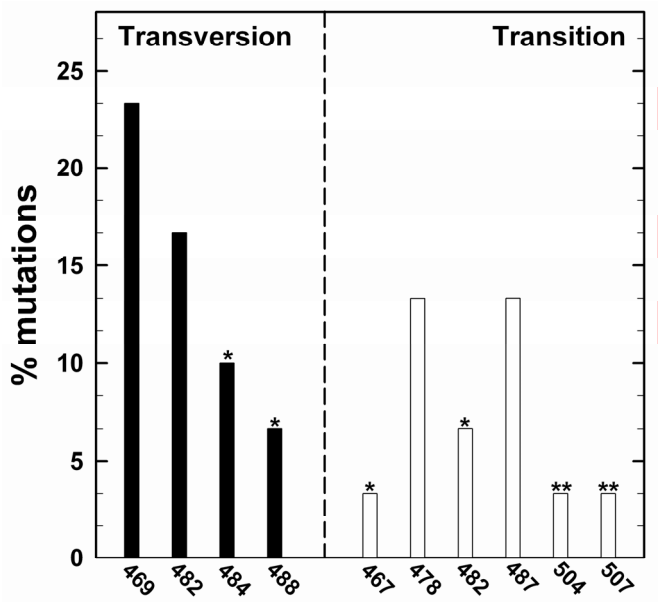


Fig. 3-20 Frequency of ionizing radiation (X-rays and accelerated HZE particle) treated or high vacuum exposed *B. subtilis* spores-induced mutations (transversion or transition mutation) determined in the sequenced part of the *rpoB* gene of *B. subtilis* 168 (for details see 2.2.8.3). Asterisk(s) marked *rpoB*-mutations were newly identified in framework of this thesis in cluster I arising from irradiated spores were detected: L467P, A478V, R484P and A488P, as well as two located codon changes R504H and H507R (labeled with two asterisks) between cluster I and II (Campbell et al., 2001).

It should be noted that all Rif^r mutations sequenced in this study were selected, purified and cultivated over several generations on NB agar plates with rifampicin concentrations of 50 µg per ml, which is 100 times higher than the wild-type *B. subtilis* 168 rifampicin resistance (Nicholson and Maughan, 2002). Therefore on those plates only Rif^r-resistant mutants were expected (and used for the analyses). For further verifying those new *rpoB* mutations, genetical transformation experiments of the described *rpoB*-Rif^r sequences in rifampicin-sensitive strains of *B. subtilis* 168 are required. After transformation, identical nucleotide substitutions from the donor strain should lead to Rif^r congressant (rifampicin-resistance in the transformants). However such experiments were not possible in the frame of this work.

3.4.3 Oxidative DNA damage formation after exposure of spores to ionizing radiation

Ionizing radiation-induced damage to the DNA is often described in terms of direct and indirect effects. The direct effect corresponds to ionization reactions of the incident particle directly with components

of the DNA. One of the main features of accelerated heavy ions is their linear energy transfer (LET), which implies that the density of ionization events along a particle (high LET) track is mucher higher than for low LET radiation, e.g. X-rays. This LET specificity was shown by an increase in the ratio between the yields of double- and single-strand breaks upon exposure of (bare) DNA to radiations of increasing LET (Roots et al., 1989). On the other hand, the indirect effect of ionizing radiation involves the reaction of DNA components with reactive species produced upon radiolysis of e.g., water molecules, among which hydroxyl radicals ($^{\bullet}$OH) are the most reactive ones (Douki et al., 2006). Such oxidative DNA damage has not yet been studied in bacterial spores after irradiation with radiations of different LET. For studying the formation of spore DNA base damage mainly caused by indirect effects of heavy ion bombardment, wild-type and major SASPs-deficient spores were irradiated with three heavy ions that were of different LET (He of 2.2 keV/µm, C of 12 keV/µm and Fe of 200 keV/µm). The irradiation procedure, sample recovery and DNA extraction out of spores are described in detail in 2.1.7, 2.2.2.1 and 2.2.4.2. Oxidized nucleosides, thymidine glycols (ThdGly) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), were quantified in DNA samples of spores exposed to heavy ions (2.3.2). Radiation doses were identical to those applied in the survival studies (Fig. 3-16). DNA damge induction rates were obtained after data normalization and substraction of the background levels. The dose effect relationships for the induction of 8-oxo-7,8-dihydro-2'-deoxyguanosine (Fig. 3-21) or of thymidine glycols (Fig. 3-22) show, within the experimental errors, linear dose effect curves.

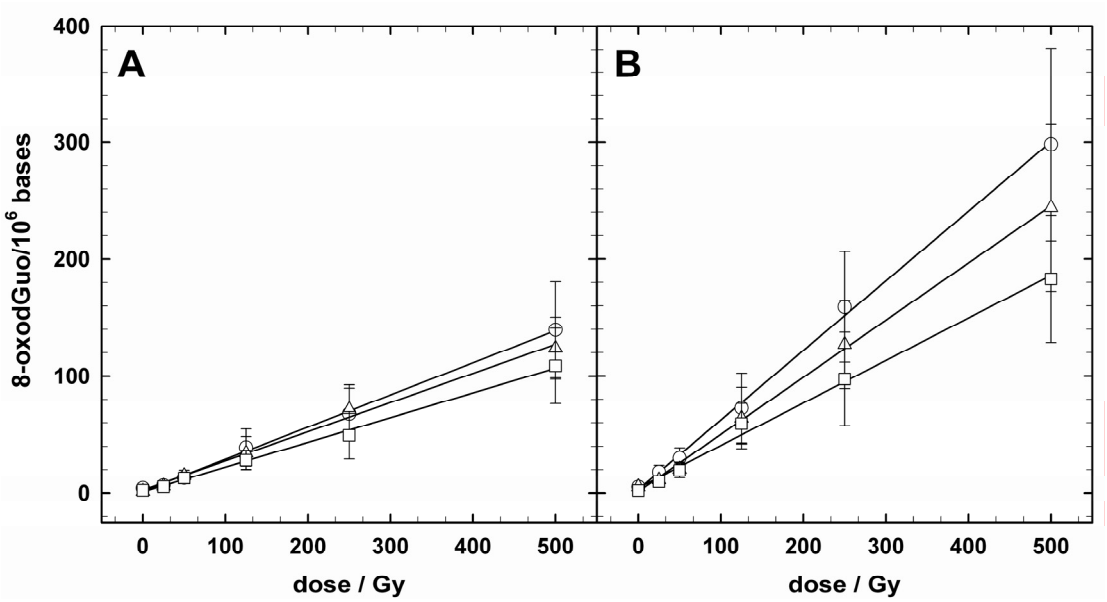


Fig. 3-21 Dose-effect curve for the formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) in the DNA of *B. subtilis* wild-type (**A**) and *sspA sspB*-deficient (**B**) spores exposed either to He (150 MeV/n, 2.2 keV/µm; open circles), C (400 MeV/n, 12 keV/µm; open triangles) or Fe (500 MeV/n, 200 keV/µm; open squares) ion beam. Data are reported as averages and standard deviations.

Table 3-18 Yields of 8-oxo-7,8-dihydro-2'-deoxyguanosine in *B. subtilis* spores exposed to accelerated heavy ions

Strain	He 150 MeV/n	C 400 MeV/n	Fe 500 MeV/n
B. subtilis (wild-type)	$(2.8 \pm 0.9)\times10^{-1}$	$(2.6 \pm 0.8)\times10^{-1}$	$(2.2 \pm 0.7)\times10^{-1}$
sspA sspB	$(6.3 \pm 2.1)\times10^{-1}$	$(4.7 \pm 1.7)\times10^{-1}$	$(3.9 \pm 1.2)\times10^{-1}$
ΔSASPs/w.t.[a]-SASPs	2.3 ± 0.5	1.8 ± 0.4	1.7 ± 0.4
w.t.[a] 8-oxodGuo / 1 chromosome[c]	1180 ± 386	1095 ± 298	927 ± 224
ΔSASPs[b] 8-oxodGuo / 1 chromosome[c]	2655 ± 784	1981 ± 681	1644 ± 473

Data are expressed as lesions/10^6 bases per Gy (if not explained otherwise).[a]w.t. = wild-type (*B. subtilis* 168 DSM 402). [b]ΔSASPs = *sspA sspB* spores (PS356). [c]Oxidative DNA damage level calculated on the chromosome size of *B. subtilis* 168 after 0.5 kGy irradiation of the respective heavy ion (according to the genomic information published by Kunst et al., 1997). Data are averages and standard deviations.

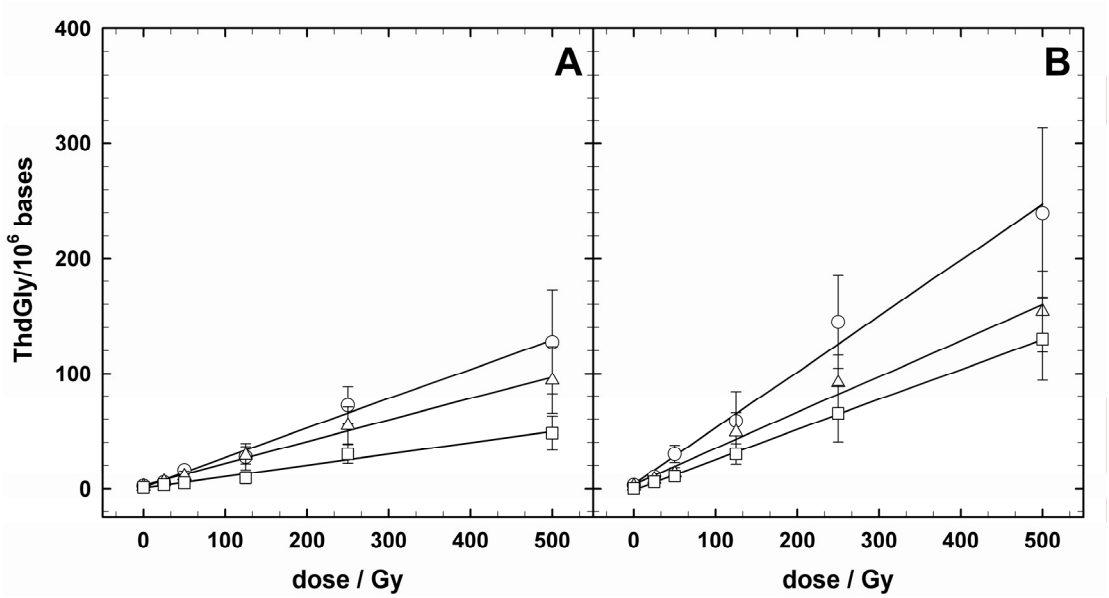


Fig. 3-22 Dose-effect curve for the formation of thymidine glycols (ThdGly) within the DNA of *B. subtilis* wild-type (**A**) and *sspA sspB*-deficient (**B**) spores exposed either to He (150 MeV/n, 2.2 keV/µm; open circles), C (400 MeV/n, 12 keV/µm; open triangles) or Fe (500 MeV/n, 200 keV/µm; open squares) ion beam. Data are reported as averages and standard deviations.

Table 3-19 Yields of thymidine glycol adducts in *B. subtilis* spores exposed to accelerated heavy ions [a]

Strain	He 150 MeV/n	C 400 MeV/n	Fe 500 MeV/n
B. subtilis (wild-type)	$(2.6 \pm 0.9)\times10^{-1}$	$(2.0 \pm 0.8)\times10^{-1}$	$(1.0 \pm 0.4)\times10^{-1}$
sspA sspB	$(5.1 \pm 1.6)\times10^{-1}$	$(3.3 \pm 1.1)\times10^{-1}$	$(2.4 \pm 0.9)\times10^{-1}$
ΔSASPs/w.t.[a]-SASPs	1.9 ± 0.6	1.7 ± 0.5	2.4 ± 0.7
w.t.[a] 8- ThdGly / 1 chromosome[c]	1091 ± 257	843 ± 203	421 ± 96
ΔSASPs[b] 8- ThdGly / 1 chromosome[c]	2149 ± 590	1391 ± 451	1012 ± 309

[a] For details on the data presentation, see Table 3-18, footnotes.

With decreasing LET of the applied ions the level of oxidized nucleosides increased as can seen in Fig. 3-21 and Table 3-18 for 8-oxodGuo and in Fig. 3-22 and Table 3-19 for ThdGly, which is in good agreement to a report of Douki et al. (2006). They observed a similar tendency in heavy ions-irradiated human monocytes and bare calf thymus DNA. In spores of both strains tested, the yield of ThdGly induced by He 150 MeV/n (LET 2.2 keV/µm) was almost twice as much as that induced by the same dose of Fe 500 MeV/n (LET 200 keV/µm) (Table 3-19). This trend was less clear for the induction of

8-oxodGuo (Table 3-18). Because this correlation of induced oxidative DNA damage with LET is opposite to that observed for inactivation (Fig. 3-16) or Rif^r mutagenesis (Fig. 3-18) other than oxidative DNA damage – probably DNA strand beakage (Micke et al. 1994) – might be mainly responsible for spore killing or mutagenesis by accelerated heavy ions.

It is interesting to note that the yield in both, 8-oxodGuo and ThdGly, was about twice as high in *B. subtilis* spores deficient in the α/β-type SASPs formation (*sspA sspB* spores) (only 20 %) than in α/β-type SASPs wild-type spores, and this for all radiation qualities applied. This increased yield in oxidative damage in irradiated *sspA sspB* spores is probably based on the absence of SASPs in DNA-SASP binding. This leads to the assumption that the α/β-type small, acid-soluble spore proteins may react as scavengers for the radiolytically formed radicals. Such a reactive oxygen species (ROS) scavenger is e.g., melanin, which is known for its role as active oxygen species protectant (Bustamante et al., 1993). Hullo et al. (2001) report of a brown melanin-like pigment synthetized by the spore coat protein CotA. They found that spores deficient in *cotA* were significantly more sensitive to terrestrial UV radiation at wavelengths > 290 nm, which is known to induce also oxidative damage to the DNA. Similarly, UV protection (UV-A > 320 nm) was obtained in strongly red-pigment *B. atrophaeus* (DSM 675) spores compared to less and non-pigmented relatives (Moeller et al., 2005). Both reports on the role of pigmentation in the spore resistance to environmentally relevant UV wavelengths suggest that those pigments – in addition to putative UV-screening - may act as antioxidants to prevent lipid peroxidation in spore membranes or as scavengers of radiation-induced ROS to avoid oxidative damage induction to the spore DNA (indirect effect). But finally, the role of the pigments in spore resistance to UV or ionizing radiation and their location within the spore remain to be determined.

3.4.4 Transcriptional profile of ionizing radiation treated *B. subtilis* spores during germination

Ionizing radiation induces DNA double-strand breaks (DSBs) that are the most lethal form of DNA damage (Hutchinson, 1985), as has also determined for bacterial spores (Micke et al., 1994). Efficient DSB repair is crucial for spore survival after ionizing radiation exposure. DSB repair is carried out by homologous recombination of chromosomal fragments in *E. coli* (Kuzminov, 1999) and several other microorganisms (vegetative cells). Astoundingly efficient DSB repair has been observed in *Deinococcus radiodurans* after exposure to high doses (> 10 kGy) of ionizing radiation, which is

achieved after short recovery times. As DNA repair mechanisms the sequential action of a RecA-independent single-strand annealing (SSA) and a RecA-dependent homologous recombination pathway has been revealed (Daly and Minton, 1996). Most of the DNA repair genes identified in *D. radiodurans* are functionally homologous to those in other prokaryotic species and the number of genes identified in *D. radiodurans* that are involved in the DNA repair is not different from the number of repair genes in *E. coli* or *B. subtilis* (Makarova et al., 2001). Therefore, the extremely high resistance of *D. radiodurans* cells as well as of *B. subtilis* spores to ionizing radiation may be attributable to still unknown repair genes (or pathways). In the case of *B. subtilis* 168 some poorly characterized "*y*"-genes might be candidates for a repair pathway leading to the high radiation resistance of their spores.

To define the repertoire of *B. subtilis* genes responding to ionizing radiation, transcriptional profiles were examined in germinating (after 1 h) spores by use of cDNA microarrays. To study the *recA*-dependency in DNA repair, wild-type spores and *recA* spores were equally treated under standardized experimental conditions, i.e. irradiated with a dose of 0.5 kGy X-rays or accelerated Fe 500 MeV/n (200 keV/µm) ions, leading to spore suvival of 43.5 or 7.8 % for wild-type spores and 2.5 or 0.4 % for *recA* spores, respectively. Spore germination was carried out under idential growth conditions i.e. 37°C and optimal vigorous aeration, as desribed previously (2.1.6). In addition, non-irradiated spores were equally treated for spore germination.

As described before (3.2.3 and 3.3.3), prior to quantification of the array data, the quality and reproducibility of the array experiment were estimated by comparing the normalized spot intensities in the scattered diagram for 60 min germinating wild-type and *recA* spores. Array data from hybridization of independent samples representing the same cultivation condition always yielded high Pearson correlation coefficient with $r \geq 0.900$, e.g., for accelerated Fe-ions irradiated spores of the wild-type (r = 0.917) and for spores of the *recA* mutant (r = 0.926) (Fig. 3-23).

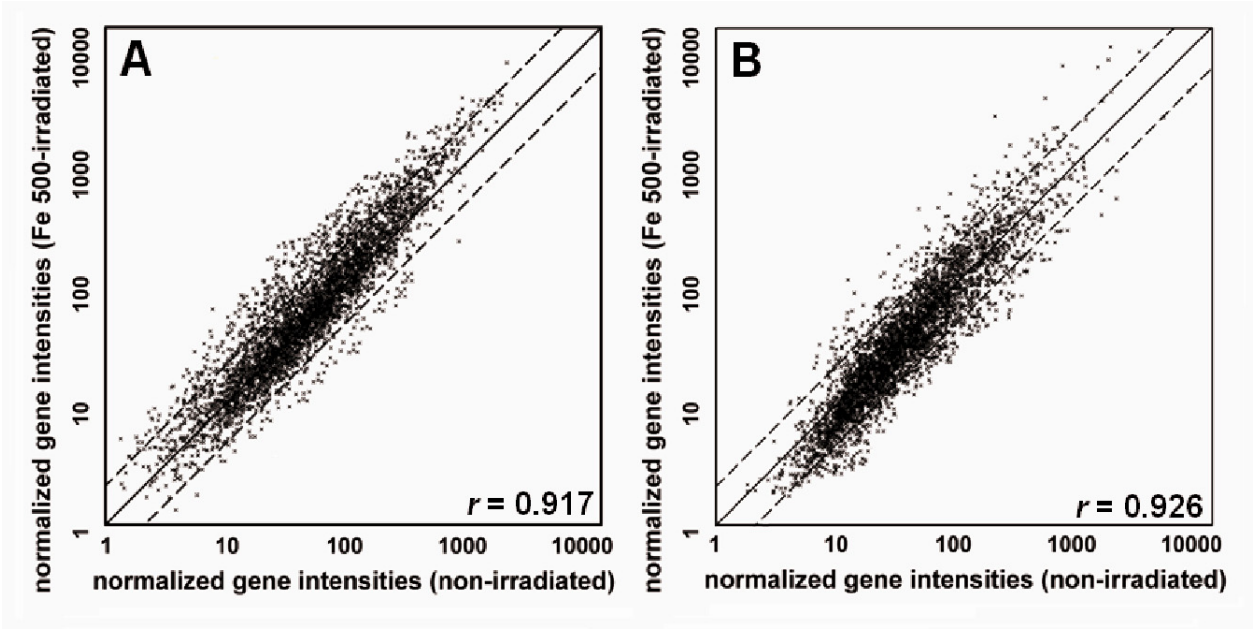


Fig. 3-23 Scatter diagrams of normalized spot intensities, exemplified for gene intensities after 60 min germination of Fe-irradiated (500 MeV/n, LET 200 keV/µm) samples plotted versus non-irradiated ones; wild-type (**A**) and *recA* (**B**) spores. The significance threshold of twofold induction or repression is symbolized by the two dashed lines. Data are expressed as mean values (n = 3). r = Pearson correlation coefficient.

By using a 2-fold balanced differential expression as the appropriate threshold level, 452 (X-rays) / 409 (accelerated Fe-ions) of the 4107 known and unknown ("*y*"-) genes were found to be up-regulated in the irradiated wild-type strain (11.0 / 9.9 %), whereas only 274 (X-rays) / 249 (Fe 500 Me/V, 200 keV/µm) genes were induced in case of the *recA* strain (6.7 / 6.1 %). In Table 3-20 the 131 up-regulated genes with a significant ≥ 2.5-fold change for X-ray irradiated wild-type spores are listed, determined from the 60 min-germinated spore population. In addition the putative LexA-binding site according to Au et al. (2005) and their response to further DNA damaging agents were determined for all 131 genes by bibliographic search (for details see footnotes in Table 3-8).

Table 3-20 Ionizing radiation-induced (X-rays[1] and accelerated Fe-ions[2]) genes after 60 min germination[a]

Gene	Function	Fold induction		LexA-bind. site	Induced by further stimulons?
		$^{1/2}$w.t.	$^{1/2}$*recA*		
recF	DNA repair and genetic recombination	5.2 / 4.8	1.5 / 1.9	+	MMC, UV
gyrB	DNA gyrase (subunit B)	2.9 / 4.1	2.0 / 1.7		

tmk	thymidylate kinase	3.5 / 2.8	2.2 / 2.8		
yabD	unknown; similar to deoxyribonuclease [*Geobacillus kaustophilus* HTA426, E value 1e-109]	6.7 / 5.9	2.3 / 2.6		SA
radA	DNA repair protein homolog	4.7 / 3.9	1.8 / 1.5		UV
yacK	unknown; similar to DNA-binding protein / DNA integrity scanning protein (DisA) [*B. licheniformis* ATCC 14580, E value 1e-172]	2.4 / 2.9	2.1 / 1.8		GS, HS
ybaJ	unknown; similar to methyltransferase [*B. cereus* G9241, E value 1e-81]	5.4 / 3.7	3.0 / 2.2		
sigW	RNA polymerase ECF-type sigma factor	4.5 / 3.7	3.1 / 2.6		
adaB	O^6-methylguanine-DNA methyltransferase	6.0 / 4.3	2.7 / 2.2		
ydcL	unknown; similar to site-specific recombinase, phage integrase family [*Enterococcus faecalis* V583, E value 5e-71]	3.8 / 2.9	1.1 / 1.2		
ydfH	unknown; similar to ATP-binding region, ATPase-like: histidine kinase, dimerisation and phosphoacceptor region [*B. weihenstephanensis* KBAB4, E value 3e-59]	3.0 / 3.2	1.8 / 2.4		
dinB	nuclease inhibitor	16.2 / 14.4	1.4 / 1.1	+	MMC, PS, UV
thiL	thiamine-monophosphate kinase	7.1 / 3.5	1.9 / 2.3		
ydiS	unknown; similar to GTPase subunit of DNA restriction endonuclease-like / type II restriction-modification system restriction subunit [*B. cereus* ATCC 14579, E value 1e-88]	3.3 / 2.8	2.4 / 2.0		
guaA	GMP synthetase	2.6 / 1.9	1.3 / 1.4		
pbuG	hypoxanthine/guanine permease	5.1 / 3.9	2.2 / 1.9		
ligA	DNA ligase (NAD-dependent)	4.7 / 6.3	1.6 / 1.2	+	MMC, UV
yefB	unknown; similar to site-specific recombinase (resolvase family) [*Geobacillus kaustophilus* HTA426, E value 3e-55]	18.9 / 11.4	1.9 / 2.5		
yefC	unknown; similar to site-specific recombinase, resolvase family [*B. cereus* G9241, E value 2e-23]	12.7 / 10.1	1.3 / 1.4		
yhcR	unknown; similar to 5'-nucleotidase / 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase bifunctional periplasmic precursor protein [*B.* sp. NRRL B-14911, E value 1e-123]	4.7 / 3.5	2.1 / 2.7		
yhaZ	unknown; similar to DNA alkylation repair enzyme [*Lactobacillus sakei* subsp. *sakei* 23K, E value 4e-80]	8.0 / 6.3	2.3 / 1.9	+	MMC, PS, UV
yhaO	unknown; similar to DNA repair exonuclease [*B. thuringiensis* str. Al Hakam, E value 3e-87]	4.4 / 3.9	2.4 / 2.2	+	MMC, PS, UV
gerPF	probable spore germination protein	2.7 / 2.8	2.6 / 3.0		
prsA	protein secretion (post-translocation molecular chaperone)	3.0 / 2.7	1.9 / 2.2		
ykoQ	unknown; similar to DNA repair exonuclease / phosphoesterase [*B. cereus* E33L, E value 6e-91]	14.4 / 9.9	3.1 / 1.8		
ykoW	unknown; similar to diguanylate cyclase/phosphodiesterase (GGDEF & EAL domains) with PAS/PAC sensor(s) [*Pseudomonas fluorescens* PfO-1, E value 5e-92]; sensory box/GGDEF family protein [*B. cereus* E33L, E value 6e-95]	6.4 / 5.7	3.6 / 3.2		
ykvT	unknown; similar to spore cortex-lytic enzyme [*Geobacillus kaustophilus* HTA426, E value 4e-28]	3.8 / 4.4	3.2 / 2.7		PS
ykwD	unknown; similar to allergen V5/Tpx-1 related [*B. licheniformis* ATCC 14580, E value 3e-77]	2.6 / 2.8	1.7 / 1.9		
ykuU	unknown; similar to alkyl hydroperoxide reductase [*B. licheniformis* ATCC 14580, E value 1e-100]	6.4 / 3.2	2.4 / 2.0		AG, SA

ykzI	unknown; similar to exonuclease [*Syntrophomonas wolfei* subsp. *wolfei* str. Goettingen, E value 1.2]	3.0 / 2.6	1.8 / 1.4		
ylbN	unknown; similar to nucleic acid-binding protein [*Lactococcus lactis* subsp. *cremoris* SK11, E value 1e-10]	4.1 / 3.2	2.0 / 1.3	+	
mraY	phospho-N-acetylmuramoyl-pentapeptide transferase	2.5 / 2.6	1.3 / 1.9		
murG	UDP-N-acetylglucosamine-N-acetyl-muramyl-(pentapeptide)-pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	3.0 / 2.8	1.7 / 2.2		GS
pyrR	transcriptional attenuation of the pyrimidine operon / uracil phosphoribosyltransferase activity	3.7 / 3.3	2.7 / 3.8		MMC, SA
gmk	guanylate kinase	2.8 / 2.1	1.6 / 1.8		
yloH	unknown; similar to DNA-directed RNA polymerase omega subunit [*B. subtilis* subsp. *subtilis* str. 168, E value 4e-30]	3.1 / 2.4	1.9 / 2.4		
yloN	unknown; similar to radical SAM family enzyme [*B. cereus* ATCC 14579, E value 1e-169]	4.4 / 3.8	1.4 / 3.0		
yloV	unknown; similar to predicted kinase related to hydroxyacetone kinase [*B. cereus* ATCC 14579, E value 0.0]; putative phosphatase [*Staphylococcus aureus* subsp. *aureus* Mu50, E value 1e-171]	2.7 / 2.2	1.9 / 1.9		
recG	probable ATP-dependent DNA helicase	4.0 / 6.1	1.8 / 1.2		MMC
ylpC	unknown; similar to transcriptional regulator of fatty acid biosynthesis [*B. subtilis subsp. subtilis* str. 168, E value 2e-87]	2.6 / 3.0	1.9 / 2.3		
pyrH	uridylate kinase	2.5 / 2.0	1.7 / 1.3		
uppS	probable undecaprenyl pyrophosphate synthetase	3.0 / 3.1	2.2 / 2.4		
polC	DNA polymerase III (alpha subunit)	6.7 / 4.4	1.6 / 1.1		
ylxR	unknown; similar to nucleic-acid-binding protein implicated in transcription termination [*Lactobacillus brevis* ATCC 367, E value 5e-16]	3.3 / 1.9	1.4 / 1.1		
recA	multifunctional protein involved in homologous recombination and DNA repair (LexA-autocleavage)	24.1 / 18.4	1.4 / 1.1	+	MMC, PS, UV
ymdA	unknown; similar to metal dependent phosphohydrolase [*Desulfitobacterium hafniense* DCB-2, E value 1e-161]	7.1 / 4.9	2.3 / 2.7		
ymaD	unknown; similar to organic hydroperoxide resistance protein OsmC, predicted redox protein, regulator of sulfide bond formation [*Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1, E value 2e-07]	8.1 / 6.4	4.7 / 3.3		PS
ymaB	unknown; similar to phosphoesterase [*B. clausii* KSM-K16, E value 7e-60]; MutT/nudix family protein [*Staphylococcus epidermidis* RP62A, E value 9e-34]	4.8 / 6.0	3.1 / 2.4		MMC
yncB	unknown; similar to Dnase / RNase endonuclease [Bacteriophage SPBc2, E value 3e-84]	2.8 / 1.7	1.3 / 1.5		
yndK	unknown; similar to excinuclease ABC subunit B [*Aquifex aeolicus* VF5, E value 2.9]	5.7 / 3.8	3.4 / 1.4		
lexA	transcriptional repressor of the SOS regulon	3.3 / 4.0	1.1 / 1.4	+	MMC, PS, UV
yneB	unknown; similar to resolvase, N-terminal domain – putative recombinase [*B. licheniformis* ATCC 14580, E value 4e-90]; site-specific recombinase [*B. cereus* ATCC 14579, E value 9e-78]	17.4 / 13.1	1.3 / 1.8	+	MMC, PS, UV

yobH	unknown; similar to UV-damage repair protein (UvrX) [UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-108]; ImpB/MucB/SamB family protein [Bacteriophage SPBc2, E value 1e-108]	8.7 / 6.1	1.9 / 1.2	+	MMC, UV
yozL	unknown; similar to YolD homolog [*B.* sp. NRRL B-14911, E value 0.009]	3.8 / 4.6	1.1 / 1.7	+	MMC, UV
yocA	unknown; similar to transposon-related protein [*Lactococcus lactis* subsp. *lactis* Il1403, E value 9e-20]; soluble lytic murein transglycosylase related regulatory protein [*Lactococcus lactis* subsp. *cremoris* SK11, E value 5e-20]	2.6 / 2.9	1.7 / 1.5		SA
yocH	unknown; similar to cell wall-binding protein [*Oceanobacillus iheyensis* HTE831, E value 5e-46]; N-acetylmuramoyl-L-alanine amidase [*Oceanobacillus iheyensis* HTE831, E value 3e-11]	4.4 / 3.7	2.6 / 1.7		SA
yocI	unknown; similar to ATP-dependent DNA helicase (RecQ) [*B. cereus* ATCC 14579, E value 0.0]	10.1 / 6.8	1.5 / 2.0		
yocJ	unknown; similar to acyl carrier protein phosphodiesterase [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-115]	2.8 / 3.1	1.9 / 1.8		
yozR	unknown; similar to heat shock protein Hsp20 [*B. weihenstephanensis* KBAB4, E value 8e-04]	6.8 / 5.0	3.3 / 2.8		
yosH	unknown; similar to DNA polymerase III, alpha subunit, gram-positive type [*C. perfringens* ATCC 13124, E value 0.84]	2.9 / 2.0	1.4 / 1.9		
yorB	unknown; similar to DNA-directed RNA polymerase beta subunit [*Treponema denticola* ATCC 35405, E value 2.4]	3.8 / 4.1	1.9 / 1.1	+	
ligB	ATP-dependent DNA ligase	4.2 / 2.9	2.1 / 3.4		
yoqL	unknown; similar to HNH endonuclease: HNH nuclease [*Prochlorococcus marinus* str. AS9601, E value 1e-04]	3.7 / 3.5	2.4 / 2.0		
yopP	unknown; similar to site-specific recombinase, phage integrase family [*Staphylococcus epidermidis* RP62A, E value 7e-20]	5.1 / 2.4	1.7 / 1.5		
yopJ	unknown; similar to ATP-dependent DNA helicase PcrA, putative [*C. perfringens* SM101, E value 0.14]	2.8 / 3.3	2.1 / 1.4		
yopC	unknown; similar to DNA polymerase zeta catalytic subunit [*Entamoeba histolytica* HM-1:IMSS, E value 0.43]	3.8 / 2.4	2.2 / 2.0		
yonH	unknown; similar to *D*-amino acid oxidase [*Candida albicans* SC5314, E value 0.43]	2.5 / 2.4	2.4 / 2.0		
yomZ	unknown; similar to MutS 2 protein [*B. weihenstephanensis* KBAB4, E value 6.1]; cyclic nucleotide-binding domain containing protein [*Tetrahymena thermophila* SB210, E value 3.6]	6.8 / 5.1	3.0 / 1.9		
yomX	unknown [Bacteriophage SPBc2]; hypothetical protein BSU21190 [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-121]	2.6 / 2.7	1.9 / 2.5		
yomO	unknown; similar to anticodon nuclease PrrC [*Flavobacterium johnsoniae* UW101, E value 0.43]	2.5 / 1.3	1.2 / 1.7		
uvrX	ImpB/MucB/SamB family protein [Bacteriophage SPBc2]; UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168 E value 0.0]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 1e-103]	7.1 / 6.3	2.3 / 1.4	+	

yolD	unknown; similar to putative chromosome segregation protein, SMC ATPase superfamily [*Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986, E value 5.1]; cyclic nucleotide-binding domain containing protein [*Tetrahymena thermophila* SB210, E value 8.7]	4.0 / 3.5	1.3 / 1.2	+	
yokA	unknown; similar to resolvase, N-terminal: recombinase [*B. weihenstephanensis* KBAB4, E value 1e-40]	7.9 / 10.1	1.9 / 1.3		
yppQ	unknown; similar to methionine sulfoxide reductase B [*B. subtilis* subsp. *subtilis* str. 168, E value 4e-82]	5.6 / 4.7	3.4 / 3.0		
degR	degradative enzyme production	2.6 / 1.8	1.4 / 1.2		
xpt	xanthine phosphoribosyltransferase	4.0 / 2.9	2.7 / 2.2		
ndk	nucleoside diphosphate kinase	6.0 / 4.1	3.7 / 2.4		
mtrA	GTP cyclohydrolase I	2.8 / 2.7	2.0 / 1.2		
hbs	non-specific DNA-binding protein HBsu	5.5 / 4.8	2.6 / 2.1	+	
ypeB	unknown; similar to spore-cortex hydrolysis, spore germination protein [*Desulfotomaculum reducens* MI-1, E value 3e-53]	3.0 / 2.7	1.9 / 1.4		
ypbG	unknown; similar to DNA repair exonuclease; phosphoesterase, N-terminus [*B. anthracis* str. Sterne, E value 1e-23]	8.7 / 7.4	3.8 / 2.1		
yqkC	unknown; similar to hypothetical protein BSU23650 [*B. subtilis* subsp. *subtilis* str. 168, E value 2e-40]	4.1 / 3.0	1.6 / 1.4	+	
yqkB	unknown; similar to molybdopterin oxidoreductase [*Alkalilimnicola ehrlichei* MLHE-1, E value 5.6]	2.5 / 1.9	1.1 / 1.2	+	
yqkA	unknown; similar to acetyltransferase YqkA [*B. licheniformis* ATCC 14580, E value 2e-82]	3.0 / 3.3	1.3 / 1.1	+	
yqjZ	unknown; similar to antibiotic biosynthesis monooxygenase [*Pseudomonas fluorescens* PfO-1, E value 3e-31]	2.6 / 2.4	1.7 / 2.0	+	MMC, UV
yqjY	unknown; similar to acetyltransferase, GNAT family [*B. anthracis* str. Ames, E value 5e-40]	3.7 / 2.1	1.4 / 1.3	+	MMC, UV
yqjX	unknown; similar to similar to YolD homolog [*B.* sp. NRRL B-14911, E value 1e-04]	4.1 / 2.3	1.4 / 2.0	+	MMC, SA, UV
yqjW	unknown; similar to DNA polymerase IV 2 (Pol IV 2), ATP/GTP-binding protein (ImpB/MucB/SamB family) [*B. halodurans* C-125, E value 1e-142]; DNA-damage repair protein [*Oceanobacillus iheyensis* HTE831, E value 1e-81]	8.1 / 6.6	1.7 / 2.7	+	MMC, SA, UV
yqjH	unknown; similar to DNA polymerase IV [*B.* sp. NRRL B-14911, E value 1e-130]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 7e-83]	7.4 / 6.0	3.1 / 2.9		
yqiZ	unknown; similar to amino acid ABC transporter, ATP-binding protein [*B. cereus* ATCC 10987, E value 4e-94]	3.3 / 2.9	2.4 / 2.3		HS
pbpA	penicillin-binding protein 2A (spore outgrowth)	3.0 / 2.8	1.8 / 1.9		
ypfS	unknown; similar to (AP, apurinic apyrimidinic) endonuclease IV [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-169]	5.0 / 3.1	2.2 / 2.3		
dnaG	DNA primase	4.2 / 5.3	2.4 / 1.7		
era	GTP-binding protein	2.5 / 2.0	1.6 / 1.5		
cdd	cytidine / deoxycytidine deaminase	3.3 / 4.1	1.9 / 1.8		
comEC	late competence operon required for DNA binding and uptake	2.6 / 2.2	2.2 / 2.1		PS

yqxJ	unknown; similar to chromosome segregation ATPase-like protein [*Trichodesmium erythraeum* IMS101, E value 0.052]; DNA double-strand break repair rad50 ATPase [*Methanococcus maripaludis* S2, E value 0.052]	5.0 / 5.4	1.9 / 2.2		SA
yqaG	unknown; similar to DNA-binding protein (helix-turn-helix) [*Burkholderia mallei* ATCC 23344, E value 3.1]	7.7 / 6.0	4.2 / 3.8		
udk	uridine kinase	2.6 / 2.3	2.4 / 2.2		
yrrK	unknown; similar to Holliday junction resolvase-like protein [*B. subtilis* subsp. *subtilis* str. 168, E value 8e-72]	10.4 / 7.6	2.0 / 1.6		
yrvM	unknown; similar to dinucleotide-utilizing enzyme [*B. clausii* KSM-K16, E value 1e-103]	3.3 / 3.2	2.4 / 2.7		
yrvE	unknown; similar to single-strand DNA-specific exonuclease (RecJ) [*B. licheniformis* ATCC 14580, E value 0.0]	5.5 / 4.7	1.2 / 1.9		
ruvB	Holliday junction DNA helicase	6.3 / 4.7	3.1 / 2.6	+	MMC, UV
ruvA	Holliday junction DNA helicase	4.1 / 2.8	2.7 / 2.1	+	MMC, UV
mreC	cell-shape determining protein	2.6 / 2.9	1.8 / 1.4		
radC	probable DNA repair protein	9.3 / 12.1	4.7 / 3.3		
pyk	pyruvate kinase	4.0 / 3.8	3.3 / 2.9		
ytxJ	unknown; similar to general stress protein [*Salinibacter ruber* DSM 13855, E value 2e-08]	3.4 / 3.5	1.8 / 1.6		GS, HS
ytxH	unknown; similar to general stress protein MutS 2 protein [*Thermoanaerobacter ethanolicus* ATCC 33223, E value 0.17]; recombination and DNA strand exchange inhibitor protein [*Thermoanaerobacter tengcongensis* MB4, E value 0.23]	5.0 / 4.7	1.4 / 1.5		GS, HS
ytxG	unknown; similar to general stress protein [*B. cereus* ATCC 14579, E value 6e-24]	2.8 / 2.3	2.0 / 2.2		GS, HS, SA
ytkD	unknown; similar to phosphohydrolase (MutT/nudix family protein) [*B. cereus* ATCC 14579, E value 1e-51]; 7,8-dihydro-8-oxoguanine-triphosphatase [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-51]	8.1 / 5.4	2.1 / 1.4		
yugI	unknown; similar to polyribonucleotide nucleotidyl-transferase (stress response) [*Lactobacillus salivarius* subsp. *salivarius* UCC118, E value 6e-17]	3.0 / 2.8	1.8 / 1.6		
yukA	unknown; similar to DNA segregation ATPase [*B. clausii* KSM-K16, E value 0.0]	2.6 / 2.0	1.7 / 1.9		AG
yuiA	unknown; similar to DnaJ-class molecular chaperone with C-terminal Zn finger domain [*Rubrivivax gelatinosus* PM1, E value 0.068]; phosphoesterase (RecJ-like) [uncultured methanogenic archaeon RC-I, E value 0.20]	4.0 / 3.3	1.6 / 2.2		PS
yumB	unknown; similar to FAD-dependent pyridine nucleotide-disulphide oxido-reductase YumB [*B. licheniformis* ATCC 14580, E value 1e-166]	3.1 / 1.8	1.5 / 1.6		
yumC	unknown; similar to FAD-dependent pyridine nucleotide-disulphide oxido-reductase YumB [*B. licheniformis* ATCC 14580, E value 1e-166]; thioredoxin reductase [*B. cereus* ATCC 14579], ferredoxin--NAD(P)(+) reductase [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-145]	2.8 / 2.0	1.1 / 1.9		
mrgA	metalloregulation DNA-binding stress protein	5.5 / 4.7	3.4 / 2.8		PS, SA^R
bdbD	thiol-disulfide oxidoreductase	3.2 / 4.0	1.9 / 2.5		

tagC	possibly involved in polyglycerol phosphate teichoic acid biosynthesis (DinR-binding site; DNA-damage inducible; *dinC*)	16.4 / 21.1	1.7 / 1.3	+	MMC, PS, UV
ywqH	unknown; similar to single-strand DNA-binding protein [*B. licheniformis* ATCC 14580, E value 2e-37]	6.3 / 8.7	2.9 / 3.3		
atpG	ATP synthase (subunit gamma)	2.6 / 2.8	2.2 / 2.4		
atpB	ATP synthase (subunit a)	3.0 / 2.1	1.9 / 1.6		
upp	uracil phosphoribosyltransferase	2.5 / 2.2	1.9 / 2.3		
pyrG	CTP synthetase	2.9 / 3.4	2.2 / 2.7		
ywhD	unknown; similar to conserved hypothetical protein [*B. cereus* E33L, E value 1e-72]; DNA primase [*Buchnera aphidicola* str. APS (*Acyrthosiphon pisum*), E value 9.1]	3.7 / 3.0	2.7 / 2.1		
ywaC	unknown; similar to GTP-pyrophosphokinase YwaC [*B. licheniformis* ATCC 14580, E value 2e-94]; pyrophosphokinase / hydrolase [*Listeria welshimeri* serovar 6b str. SLCC5334, E value 1e-58]	3.7 / 4.0	1.9 / 2.2		GS
cydA	cytochrome bd ubiquinol oxidase (subunit I)	3.0 / 3.3	1.5 / 1.6		
yxkF	unknown; similar to fis-type helix-turn-helix domain protein [*B. cereus* ATCC 10987, E value 8e-24]; DNA-binding protein, Fis family [*Enterococcus faecalis* V583, E value 5e-15]	4.0 / 4.7	3.1 / 2.4		SA
ppaC	inorganic pyrophosphatase	2.6 / 2.0	1.8 / 1.8		
ssb	single-strand DNA-binding protein	20.1 / 16.3	4.6 / 5.9		MMC, UV
yyaF	unknown; similar to GTP-dependent nucleic acid-binding protein EngD [*B. licheniformis* ATCC 14580, E value 1e-177]	2.8 / 2.9	1.9 / 2.3		

[a] For details on the data presentation, see Table 3-8, footnotes [a] through [e].

To interpret the functions of the transcriptional activities after 60 min germination of the irradiated spores, seven gene ontology (GO) groups were identified by using bibliographic search (see chapter 2.4, 2.4.4 - 2.4.6), mainly involved in DNA repair, recombination, replication, nucleotides and adaptation processes, respectively. They are listed in Table 3-21 according to their functional affiliation to those GO groups.

Table 3-21 Highly ionizing radiation induced genes and their functional affiliation in 7 GO groups[a]

GO 1. DNA repair and modification/restriction

wild-type[1]: *adaB, dinB, lexA, radA, radC, tagC (dinC), uvrX, yacK (disA), ydiS, ykoQ, ykzI*•*, yhaO, yhaZ, ymaB, yndK*•*, yobH, ypbG, yqfS, yqjH, yqjW, yqxJ*•*, ytkD*

wild-type[2]: *adaB, dinB, lexA, radA, radC, tagC (dinC), uvrX, yacK (disA), ydiS, ykoQ, ykzI*•*, yhaO, yhaZ, ymaB, yndK*•*, yobH, ypbG, yqfS, yqjH, yqjW, yqxJ*•*, ytkD*

recA[1]: *adaB, radC, ykoQ, ymaB, yndK*•*, ypbG, yqjH*

recA[2]: *radC, yqjH, yqjW*

GO 2. DNA recombination

wild-type[1]: *recA, recF, recG, ruvA, ruvB, yabD, ydcL, yefB, yefC, yneB, yocI, yokA, yopP, yrrK, yrvE*

wild-type[2]: *recA, recF, recG, ruvA, ruvB, yabD, ydcL, yefB, yefC, yneB, yocI, yokA, yrrK, yrvE*

recA[1]: *ruvA, ruvB*

recA[2]: *ruvB, yabD, yefB,*

GO 3. DNA replication

wild-type[1]: *dnaG, ligA, ligB, polC, ssb, yopC*•, *yopJ*•, *yosH*•, *ywhD*•, *ywqH*

wild-type[2]: *dnaG, ligA, ligB, polC, ssb, yopJ*•, *ywhD*•, *ywqH*

recA[1]: *ssb, ywhD*•, *ywqH*

recA[2]: *ligB, ssb, ywqH*

GO 4. DNA packaging and segregation

wild-type[1]: *gyrB, hbs, yolD*•, *yozL*•, *yqjX, yukA*

wild-type[2]: *gyrB, hbs, yolD*•, *yozL*•

recA[1]: *hbs*

recA[2]: -

GO 5. Nucleotides, nucleobases, nucleotide binding and nucleic acid metabolism

wild-type[1]: *cdd, era, gmk, guaA, mtrA, ndk, pbuG, pyk, pyrG, pyrH, pyrR, tmk, udk, upp, xpt, yhcR, ykoW, ylbN, ylxR, yncB, yomO*•, *yomZ*•, *yoqL, yqaG*•, *yrvM, yxkF, yyaF*

wild-type[2]: *cdd, mtrA, ndk, pbuG, pyk, pyrG, pyrR, tmk, xpt, yhcR, ykoW, ylbN, yomZ*•, *yoqL, yqaG*•, *yrvM, yxkF, yyaF*

recA[1]: *ndk, pyk, pyrR, xpt, ykoW, yomZ*•, *yqaG*•, *yxkF*

recA[2]: *pyk, pyrG, pyrR, tmk, yhcR, ykoW, yqaG*•, *yrvM*

GO 6. Adaptation, detoxification and response to stress (ionizing radiation stimulus)

wild-type[1]: *bdbD, mrgA, ykuU, ymaD, yonH*•, *yozR, yppQ, yqiZ, yqkB*•, *ytxG, ytxH, ytxJ, yugI, yuiA*•, *yumB, yumC*

wild-type[2]: *bdbD, mrgA, ykuU, ymaD, yozR, yppQ, yqiZ, ytxH, ytxJ, yugI, yuiA*•

recA[1]: *mrgA, ymaD, yozR, yppQ*

recA[2]: *bdbD, mrgA, ymaD, yozR, yppQ,*

GO 7. Other functions

GO 7.1 Germination and cell envelope

wild-type[1]: *gerPF, murG, pbpA, ykvT, yocA, yocH, ypeB*

wild-type[2]: *gerPF, murG, pbpA, ykvT, yocA, yocH, ypeB*

recA[1]: *gerPF, ykvT, yocH*

recA[2]: *gerPF, ykvT*

GO 7.2 Bioenergetics

wild-type[1]: *atpB, atpG, cydA*

wild-type[2]: *atpG, cydA*

recA[1]: -

recA[2]: -

GO 7.3 Other functions, or/and unknown and hypothetical proteins

wild-type[1]: *comEC, degR, mraY, mreC, ppaC, prsA, sigW, thiL, uppS, ybaJ, ydfH, ykwD, yloH, yloN, yloV, ylpC, ymdA, yocJ, yomX, yorB•, yqjY, yqjZ, yqkA, yqkC, ywaC*

wild-type[2]: *mraY, mreC, prsA, sigW, thiL, uppS, ybaJ, ydfH, ykwD, yloN, ylpC, ymdA, yocJ, yomX, yorB•, yqkA, yqkC, ywaC*

recA[1]: *sigW, ybaJ*

recA[2]: *sigW, yloN, ymdA, yomX*

• Gene with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). For details on the gene function, see Table 3-20. [a] For details on the data presentation, see Table 3-9. Ionizing radiation exposure: [1] X-rays and [2] accelerated Fe-ions (500 MeV/n).

Twenty-four genes implicated in DNA repair processes were found to be up-regulated, such as the SOS response (e.g. *dinB* and *dinC*), recombination-mediated repair (e.g. *recA*), base excision (e.g. *ypbG*, a predicted *B. anthracis* str. Sterne-like DNA repair exonuclease) and oxidative stress response (e.g. *mrgA*). Especially, genes involved in the recombination-mediated repair, particularly *recA*, *recF* and *recG* (ATP-dependent DNA helicase) as well as single-stranded DNA binding protein (*ssb*), as major enzymes for the initiation of the recombination-mediated repair were significantly up-regulated (≥ 4-fold). After 1h spore germination *ssb* and *recA* were the dominant genes after irradiation by X-rays as well as by Fe ions, i.e. their up-regulation was independent of the quality of the applied ionizing radiation.

Additional to the highly expressed *rec*-genes, the following genes were highly up-regulated: seven genes encoding for DNA polymerases (four *dinB*-like DNA polymerases: *uvrX*, *yobH, yqjH* and *yqjW*; and three DNA polymerases type III: *polC*, *yosH* and *yopC*), five DNA helicase encoding genes (*recG*, *yocI* (an ATP-dependent DNA helicase (RecQ) similar to *B. cereus* ATCC 14579), *yopJ* (a putative *C. perfringens* SM101 ATP-dependent DNA helicase-like PcrA) and the two Holliday junction DNA helicases (*ruvA* and *ruvB*), two known ATP- and NAD- dependent DNA ligases (*ligA* and *ligB*) as well as eight genes encoding for DNA-binding proteins: *yacK* (DNA-binding protein / DNA integrity scanning protein (DisA), similar to *B. licheniformis* ATCC 14580), *hbs* (non-specific DNA-binding protein), *mrgA* (metalloregulation DNA-binding stress protein), *ywqH* (a putative *B. licheniformis* ATCC 14580 single-strand DNA-binding protein), *yxkF* (a predicted Fis family DNA-binding protein, similar to *Enterococcus faecalis* V583), *ssb*, *ylbN* (a probable nucleic acid-binding protein, similar to *Lactococcus lactis* subsp. *cremoris* SK11), *ylxR* (a nucleic-acid-binding-like protein, similar *Lactobacillus brevis* ATCC 367) and *yyaF* (a *B. licheniformis* ATCC 14580-like GTP-dependent nucleic acid-binding protein EngD) ensuring DNA integrity and stability (detailed information can be seen in Table 3-20).

Both *B. subtilis rad*-genes (radiation-inducible DNA repair proteins): *radA* and *radC* were highly induced after exposure of the spores to X-rays or accelerated Fe-ions. Sixteen up-regulated genes were assigned to the ionizing radiation stress response and detoxification gene ontology group (Table 3-21, GO 6.); especially the previously UV-A induced *ymaD*, a predicted hydroperoxide resistance protein (Table 3-13) was significantly induced after both types of irradiations. Futher on, *ykuU* - a *B. licheniformis* ATCC 14580-like alkyl hydroperoxide reductase, the two predicted FAD-dependent pyridine nucleotide-disulphide oxido-reductases *yumB* and *yumC* (similar to *B. licheniformis* ATCC 14580), the sulfoxide reductase *B. licheniformis* ATCC 14580-homolog *yppQ* and the *ytxJHG*-operon (a general stress regulon controlled by σ^B) exhibited high levels of transcriptional activation.

Only a few of the pathways that might be involved in direct damage reversal or base excision repair responded to ionizing irradiation. Activation of genes, involved in DNA mismatch repair and recognition were also observed. Two mutT/nudix-like genes, *ytkD* and *ymaB* were found to be up-regulated: similar observations were made after UV-A exposure of the spores and 60 min germination,

implicating an essential role of their products in the DNA mismatch repair as putative nucleotide mismatch recognition proteins. DNA mismatch repair prevents cells from manifesting DNA base content modifications (mainly A-G mismatch removal), which may lead to mutagenesis (Aravind et al., 2000). It is also interesting to note that the recently described apurinic and apyrimidinic endonuclease IV (AP) encoding gene *yqfS* (Salas-Pacheco et al., 2005) was found to be up-regulated during germination of irradiated spores (Table 3-20). By this mechanism, DNA depurination will indirectly result in additional DNA single-strand breaks as a consequence of ionizing irradiation.

Overall the majority of the DNA repair genes ensure the genome integrity and the successful DNA repair of strand-breaks. Also there were six up-regulated "*y*"-genes, which encode site-specific recombinases/resolvases: *yefB* (similar to *Geobacillus kaustophilus* HTA426), *yefC* (a *B. cereus* G9241 homolog), *yneB* (similar to *B. cereus* ATCC 14579), *yokA* (a *B. weihenstephanensis* KBAB4), *yrrK* (a putative Holliday junction) and *ydcL* (similar to a site-specific recombinase/phage integrase of *Enterococcus faecalis* V583).

Furthermore, four DNA repair/single-strand exonucleases (*yhaO*, *ykoQ*, *ypbG* and *yrvE*), similar to other *Bacillus* species, were found to be significantly induced during germination of wild-type spores that were exposed to ionizing radiation.

Twenty-seven genes involved in nucleotide binding and nucleic acid metabolism were up-regulated, implicating their role in the replacement of radiation-damaged nucleotides and new synthesis of adequate substitutes. Four nucleotide kinases (*ndk*, *pyk*, *tmk* and *udk*) and seven genes encoding guanine phosphorylation i.e. GTP- (*ydiS*, *mtrA*, *era*, *ywaC*, *yyaF* and *yqjW*) and GMP- (*guaA*) binding reactions stand out, suggesting their role in the repair of damaged guanine, e.g., 8-oxo-7,8-dihydro-2'-deoxyguanosine.

In addition, an increased induction of three known (*gerPF*, *murG* and *pbpA*) genes and four putative (*ykvT*, *yocA*, *yocH* and *yqeB)* genes encoding for spore cortex-lytic encoding enzymes and/or cell wall-binding proteins was observed. A similar gene expression of those genes and others involved in cell envelopment was obtained after UV irradiation (Tables 3-8 and 3-13) or high vacuum exposure (see

3.5) of spores. This suggests the spore peptidoglycan hydrolysis and cortical fragment lysis were also triggered under the influence of ionizing radiation. One explanation might be a secondary non-DNA damage induced by ionizing radiation, such as oxidation of spore coat components, which function as germination receptors.

Concerning the *recA* spores, the five most strongly induced genes after ionizing irradiation and 1 h germination were *ssb*, *radC*, *ymaD*, *ypbG* and *ykoW* (a putative diguanylate cyclase / phosphodiesterase), whereas the genes closely involved in DNA repair and homologous recombination (and recombination-mediated repair) e.g. *recA*, *ssb*, *yneB*, *tagC* (former *dinC*) and *yefB* were the most highly up-regulated genes in the wild-type spores (Table 3-20).

To confirm the gene expression information based on the microarray data after exposure of spores to ionizing radiation (as can seen in Table 3-20), the highly induced putative recombinase/N-terminal resolvase *yneB* (similar to *B. licheniformis* ATCC 14580) was selected for RT-PCR analysis (2.4.7). The up-regulation values after 1 h germination were 10.4 after UV-C irradiation (Table 3-8), 9.7 after UV-A irradiation (Table 3-13), 17.4/13.1 after ionizing irradiation (Table 3-20) and 11.3 after 450 d exposure to high vacuum (see 3.5). Fig. 3-24 shows the DNA bands of the amplified cDNA after RT-PCR. The amplified cDNA band for the estimated RT-PCR product was in the expected product size (241 bp).

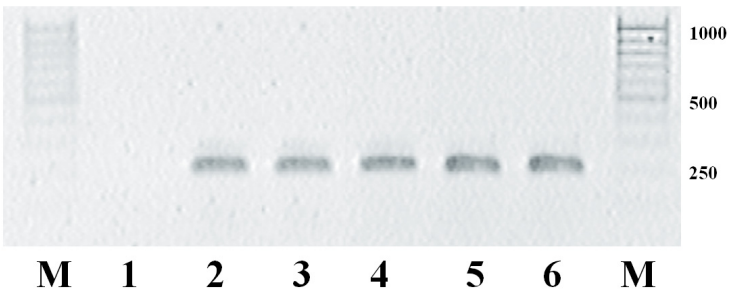


Fig. 3-24 RT-PCR analysis of wild-type *B. subtilis* 168 *yneB* - transcript. The rows are loaded with non- -irradiated control (1), X-rays irradiated (2), He (150 MeV/n, LET 2.2 keV/µm) irradiated (3), Fe (500 MeV/n, LET 200 keV/µm) irradiated (4), UV-C irradiated (5), UV-A irradiated (6) samples; M served as standard DNA marker (Hyper Ladder IV, Bioline GmbH, Luckenwalde, Germany).

Similar cDNA band patterns were obtained for the genes *ykoQ*, *yefB*, *yefC* and *yobH* from RNA samples isolated from X-ray and accelerated heavy ions (He 150 MeV/n and Fe 500 MeV/n) irradiated

spores after 60 min of germination (detailed gene functional characterization and ontology can be seen in Tables 3-20 and 3-21).

3.5 Effects of vacuum in dormant *B. subtilis* spores

The effects created by exposing bacteria to vacuum have long been a subject of interest to biologists, especially the use of vacuum in storing microorganisms as essential part of lyophilization preservation techniques (Morelli, 1962). Furthermore, there has been considerable speculation about whether or not spores and similar life forms can survive the rigors of travel through interplanetary space (Arrhenius, 1903; Brueschek et al., 1961; Horneck et al., 1994a; Nicholson et al., 2000). Experimental study of the effects of the space environment on microorganisms is one of the most important tasks of exobiology. Experiments with vacuum-exposed bacteria are useful in at least two aspects: (i) in the basic research to study the role of hydration in biological and biochemical functions of the respective tested (micro-)organism - by removing the water, and (ii) in the applied research to estimate the chances of survival of miroorganisms under space environmental conditions, which are governed among others by high vacuum.

If the pressure reaches values below the vapour pressure of a certain material, the material's surface atoms or molecules vaporize. Dehydration is the main process affecting biological material exposed to vacuum. Water plays a decisive part in the maintenance of the native conformation of the biomacromolecules, especially the DNA. Below the vapour pressure of water, with decreasing pressure the hydration water from the grooves is removed, thereby changing the structure of the DNA double helix. In vacuum-treated cells of *Escherichia coli*, less than 1 water molecule per nucleotide is available (Reitz, 1972; Schwager, 1973). The consequence of this nearly complete loss of hydration water is a partial denaturation of the DNA, which is a reversible process (Falk et al., 1962; 1963a; 1963b). Although bacterial spores excel already by low water content, under vacuum conditions the remaining water molecules surrounding the DNA and maintaining its structure are forcibly extracted, leaving DNA damage that is lethal when not repaired. Dose et al. (1992) reported of crosslinking between DNA and proteins in vacuum-exposed spores of *B. subtilis* which may finally lead to DNA single and double strand breaks (Dose and Gill, 1995). Furthermore, these structural changes of the

spore DNA, caused by vacuum forced dehydration, may also affect the photochemical and photobiological properties of vacuum-treated spores (reviewed in Bücker et al., 1972; Horneck, 1993).

3.5.1 Survival of and mutation induction in spores of *B. subtilis* exposed to high vacuum

To study the effects to high vacuum similar to outer space condtions ($P \leq 10^{-6}$ Pa), wild-tye and *recA* spores of *B. subtilis* 168 were exposed to high vacuum ($P = 10^{-7}$ Pa) for up to 30 d or 450 d, assayed for survival (Fig. 3-25A) and Rif^r mutation induction (Fig. 3-25B), and their transcriptional response to vacuum exposure was determined after 60 min of germination.

Wild-type spores of *B. subtilis* survived vacuum ($P = 10^{-7}$ Pa) exposure over 15 d or 30 d without significant inactivation (for comparison survival of *recA* spores was reduced by 2 orders of magnitude after 30 d vacuum exposure, data shown in 3.7.2). After 15 months storage in high vacuum 20 % of the wild-type spores were still capable of forming colonies on NB agar plates (Fig 3-25A).

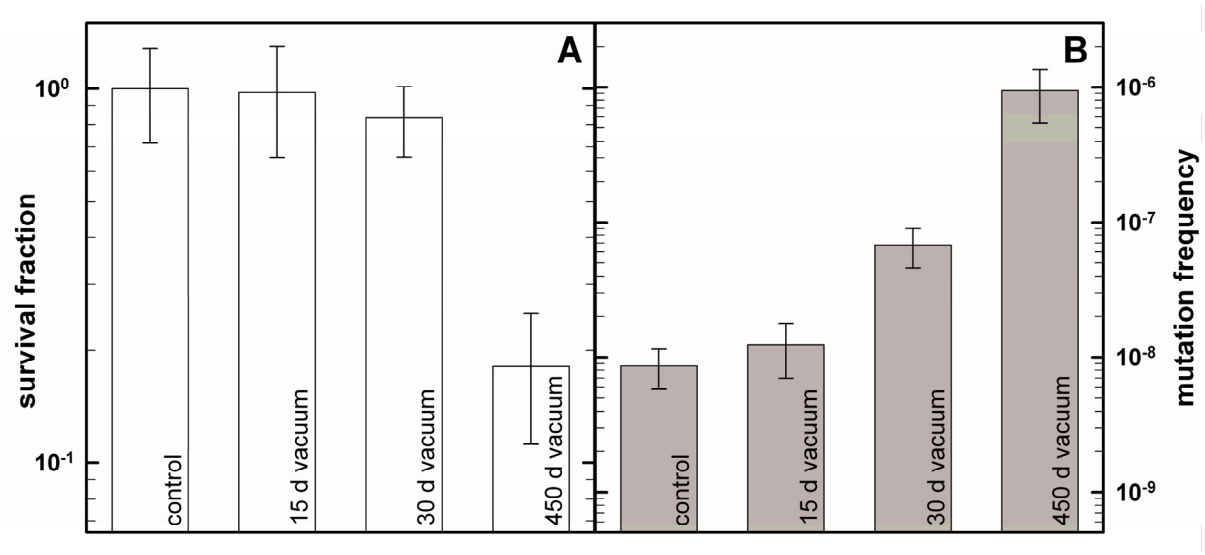


Fig. 3-25 Survival (**A**) and Rif^r mutation frequency (**B**) of *B. subtilis* 168 wild-type spores exposed to high vacuum (10^{-7} Pa). Data are averages +/- standard deviation ($n = 3$).

As alredy reported earlier, the spontaneous Rif^r mutation rate was 4.2×10^{-9}. With increasing time of exposure to vacuum the Rif^r mutation rate increased (Fig 3-25B), reaching a more than 100 times higher mutation frequency (8.9×10^{-7}) after 450 d of exposure to high vacuum. A similar mutagenic

effect of vacuum exposure has been reported by Munakata et al. (1997) for the induction of reistance to nalidixic acid. Nine Rif^r mutants were selected for detailed analysis of their location in the *rpoB* (cluster I), which was performed together with Rif^r mutants induced by ionizing radiation (Table 3-17). Six different mutational changes in the cluster I of *rpoB* were determined for spores exposed for 15, 30 or 450 d to high vacuum (Table 3-17). They were either transversions (A to T) in codon Q469L, (A to C) in codon H482P) or (G to C) in codon A488P, or they were transitions (C to T) in codon A478V or codon S487L or (G to A) in codon H507R, the latter lying outside of cluster I. However, no real mutational hot spots were identified after exposure of spores to high vacuum. The mutations were evenly spread over the cluster I of the *rpoB* gene with a maximum of two identical base changes. Detailed information on the spectrum and distribution of the Rif^r-mutations induced by vacuum or ionizing radiation is given in Table 3-17, Fig. 3-19 and 3-20.

Because storage under vacuum is currently one of the mostly used techniques for microbial preservation, the observed mutagenicity of vacuum treatment to an increased resistance to antibiotics may have serious consequences and needs further investigations, above all in stock culturing of microorganisms. Similar observations as in this study were made by Munakata et al. (1997) and Maughan et al. (2004). Furthermore, the present observation provides additional constraint to long-term survival of bacterial spores in space environment where an extremely high vacuum (10^{-7}-10^{-14} Pa: Horneck, 1993; Nicholson et al., 2000, 2005) is prevalent. The data indicate that the DNA, the carrier of the genetic information, is one of the critical targets in vacuum-induced mutagenesis.

3.5.2 Transcriptional profile of vacuum exposed *B. subtilis* spores after 1 h germination

To study the gene activation of *B. subtilis* genes responding to long-term high vacuum exposure, transcriptional profiles were recorded in germinating wild-type and *recA* spores after 1 h germination by using cDNA microarrays, as can be seen in detail in chapter 2.3 (2.3.4 - 2.3.3). For the quantification of the array data, monitoring the quality and reproducibility of the array experiments scatter diagrams for 60 min germinating wild-type spores (30 and 450 d vacuum storage) are shown in Fig. 3-26 (for further details see 3.2.3, 3.3.3 and 3.4.4). Array data from hybridization of independent samples representing the same cultivation condition always yielded a high Pearson correlation coefficient with $r \geq 0.900$ (wild-type: 0.958 / 30 d and 0.915 / 450 d).

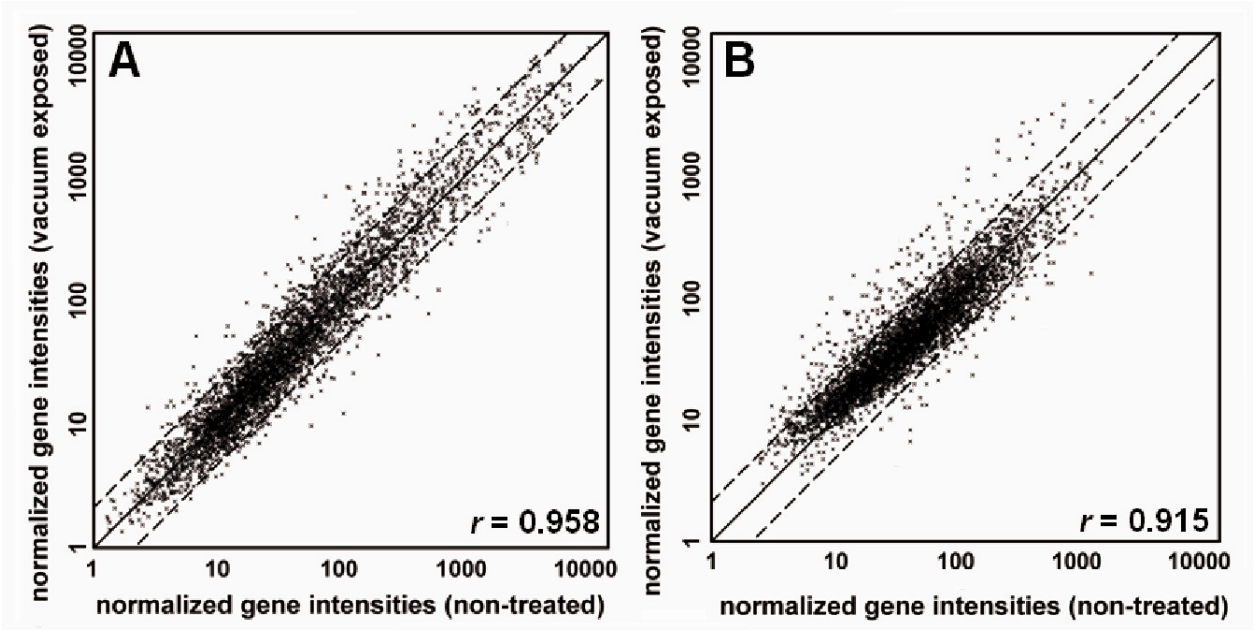


Fig. 3-26 Scatter diagrams of normalized spot intensities. Gene intensities are plotted of vacuum-exposed (**A**: 30 and **B**: 450 d) and non-treated wild-type spores after 60 min germination. The significance threshold of twofold induction or repression is symbolized by the two dashed lines. Data are expressed as mean values (n = 3). r = Pearson correlation coefficient.

Using a 2-fold cut-off value as appropriate threshold level, 276 (after 450 d; 6.7 %) and 154 (after 30 d; 3.7 %) of the 4107 known and unknown genes were found to be up-regulated in the wild-type spores, whereas only 107 (2.6 %) of the *recA* spores were induced above (or equal to) the required threshold. Table 3-22 lists the 119 highly up-regulated genes (with a significant ≥ 2.5-fold change), determined from the 60 min-germinated wild-type spore population (after 30 d vacuum). In addition for all those genes the regulation after 450 d vacuum exposure and that in the *recA* strain after 30 d vacuum exposure are given; further their putative LexA-binding site according to Au et al. (2005) and their response to further DNA damaging agents, e.g. mitomycin C were determined by bibliographic search (for detailed information see footnotes in Table 3-8).

Table 3-22 High vacuum-induced genes (after 30[1] and 450[2] d exposure) after 60 min germination[a]

Gene	Function	Fold induction		LexA-bind. site	Induced by further stimulons?
		[1/2]w.t.	[1]*recA*		
recF	DNA repair and genetic recombination	4.1 / 6.7	1.2	+	MMC, UV
dnaX	DNA polymerase III (gamma and tau subunits)	3.0 / 2.5	1.6		
purr	transcriptional repressor of the purine operons	4.1 / 2.4	2.0		

yacK	unknown; similar to DNA-binding protein / DNA integrity scanning protein (DisA) [*B. licheniformis* ATCC 14580, E value 1e-172]	3.0 / 3.2	1.7		GS, HS
rplX	ribosomal protein L24 (BL23) (histone-like protein HPB12)	3.2 / 3.5	1.9		
adk	adenylate kinase	2.6 / 2.0	1.2		
sigW	RNA polymerase ECF-type sigma factor	2.8 / 3.3	2.4		
adaB	O^6-methylguanine-DNA methyltransferase	3.1 / 2.8	1.9		
ybcS	unknown; similar to hypothetical protein BSU01930 [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-103]	8.0 / 6.0	1.3		
ycbN	unknown; similar to ABC transporter (ATP-binding protein, bacitracin ABC transporter) [*B. licheniformis* ATCC 14580, E value 1e-108]	3.0 / 2.5	2.1		
ycbR	unknown; similar to stress protein [*Psychrobacter cryohalolentis* K5, E value 5e-35]; DNA helicase, ATP-dependent dsDNA / ssDNA exonuclease V subunit [*Yersinia pestis* KIM, E value 0.64]	2.6 / 4.1	1.8		
ydcP	unknown; similar to molecular chaperone DnaK2, heat shock protein hsp70-2 [*Prochlorococcus marinus* str. MIT 9303, E value 2.8]; DNA topoisomerase I [*Synechococcus* sp. RS9917, E value 2.8]	2.9 / 5.0	2.3		
dinB	nuclease inhibitor	8.1 / 10.0	1.5	+	MMC, PS, UV
thiL	probable thiamine-monophosphate kinase	4.0 / 3.4	1.9		
ydiO	unknown; similar to DNA-methyltransferase (cytosine-specific) [*Rhodopseudomonas palustris* BisB18, E value 3e-32]	3.3 / 3.1	2.0	+	
ligA	DNA ligase (NAD-dependent)	3.8 / 4.8	1.4	+	MMC, UV
yefB	unknown; similar to site-specific recombinase (resolvase family) [*Geobacillus kaustophilus* HTA426, E value 3e-55]	10.1 / 9.1	1.8		
yefC	unknown; similar to site-specific recombinase, resolvase family [*B. cereus* G9241, E value 2e-23]	7.1 / 9.4	1.3		
yeeD	unknown; similar to DNA repair protein Rad50 [*Entamoeba histolytica* HM-1:IMSS, E value 0.62]	3.4 / 5.2	1.6		
ecsB	ABC transporter (membrane protein)	2.7 / 2.8	2.3		
yhjJ	unknown; similar to oxidoreductase, Gfo/Idh/MocA family [*B. thuringiensis* str. Al Hakam, E value 1e-111]	4.1 / 3.2	1.4		
yhjK	unknown; similar to HAD superfamily hydrolase [*B. thuringiensis* str. Al Hakam, E value 2e-88]	2.5 / 2.4	2.8		
yhjL	unknown; similar to pyridoxal phosphate-dependent enzyme apparently involved in regulation of cell wall biogenesis [*Thermoanaerobacter tengcongensis* MB4, E value 2e-60]	2.7 / 3.0	1.9		
yhjM	unknown; similar to purine nucleotide synthesis repressor [*Erwinia carotovora* subsp. *atroseptica* SCRI1043, E value 1e-37]	2.5 / 3.0	1.5		
yirY	unknown; similar to DNA repair exonuclease [*Symbiobacterium thermophilum* IAM 14863, E value 1e-151]; ATPase involved in DNA repair [*Thermoanaerobacter tengcongensis* MB4, E value 1e-145]	7.8 / 13.2	3.9		
gerPF	probable spore germination protein	3.2 / 4.4	1.9		

yjcG	unknown; similar to 2'-5' RNA ligase [*B. cereus* ATCC 14579, E value 7e-55]	2.6 / 2.9	2.3		
ykkC	unknown; similar to molecular chaperone / chaperonin [*B. halodurans* C-125, E value 3e-15]	2.7 / 2.0	1.4		
ykuL	unknown; similar to DNA polymerase type I [*Bacteroidetes* bacterium endosymbiont of *Bemisia tabaci*, E value 4.4]	3.0 / 4.8	1.8		AG, SA
murG	UDP-N-acetylglucosamine-N-acetyl-muramyl-(penta-peptide)pyrophosphoryl-undecaprenol N-acetylglucos-amine transferase	2.8 / 3.1	2.0		GS
yloC	unknown; similar to ATP-dependent DNA helicase (RecG) [*Saccharophagus degradans* 2-40, E value 1e-31]; uncharacterized stress-induced protein [*B. anthracis* str. A2012, E value 1e-61]	5.1 / 3.4	1.4		
gmk	guanylate kinase	2.6 / 3.2	1.6		
yloV	unknown; similar to kinase related to hydroxyacetone kinase [*B. cereus* ATCC 14579, E value 0.0]; phosphatase [*Staphylococcus aureus* subsp. *aureus* Mu50, E value 1e-171]	2.9 / 4.0	2.2		
clpY	two-component ATP-dependent protease [ClpQ]	3.0 / 3.5	2.1		GS, HS
tsf	elongation factor Ts	2.5 / 2.3	1.9		
pyrH	uridylate kinase	2.9 / 2.0	2.2		
cdsA	phosphatidate cytidylyltransferase	2.8 / 3.0	1.6		
recA	multifunctional protein involved in homologous recombination and DNA repair (LexA-autocleavage)	14.3 / 19.4	1.7	+	MMC, PS, UV
ymzC	unknown; similar to exonuclease, RNase T and DNA polymerase III [*Enterobacter* sp. 638, E value 3.2]	2.8 / 3.7	2.3		
lexA	transcriptional repressor of the SOS regulon	2.8 / 4.1	1.2	+	MMC, PS, UV
yneB	unknown; similar to resolvase, N-terminal domain – putative recombinase [*B. licheniformis* ATCC 14580, E value 4e-90]; site-specific recombinase [*B. cereus* ATCC 14579, E value 9e-78]	7.1 / 11.4	1.7	+	MMC, PS, UV
yoeB	unknown; similar to DNA-3-methyladenine glycosylase I [*Dictyostelium discoideum* AX4, E value 1.2]; DNA topoisomerase III [*Listeria monocytogenes* str. 4b H7858, E value 3.6]	5.0 / 6.7	2.3		GS, PS, SA^R
yozJ	unknown; similar to DNA helicase [*C. tetani* E88, E value 1.1]	3.0 / 2.4	1.2		
yozK	unknown; similar to UV-damage repair protein (uvr) [*B. subtilis subsp. subtilis* str. 168]; ImpB/MucB/SamB family protein [Bacteriophage SPBc2, E value 7e-58]	2.8 / 4.8	1.6	+	MMC
yozL	unknown; similar to YolD homolog [*B.* sp. NRRL B-14911, E value 0.009]	3.3 / 4.0	1.1	+	MMC
yocI	unknown; similar to ATP-dependent DNA helicase (RecQ) [*B. cereus* ATCC 14579, E value 0.0]	8.0 / 7.4	1.9		
yotB	unknown; similar to metallophosphoesterase [*Geobacter lovleyi* SZ, E value 5e-10]	2.7 / 3.5	1.6		
yosL	unknown; similar to DNA polymerase III, beta subunit [*Acidiphilium cryptum* JF-5, E value 1.7]	3.0 / 4.1	2.4		
yosH	unknown; similar to DNA polymerase III, alpha subunit, gram-positive type [*C. perfringens* ATCC 13124, E value 0.84]	4.4 / 4.9	2.0		

yorS	unknown; similar to 5'(3')-deoxy-ribonucleotidase [*Robiginitalea biformata* HTCC2501, E value 4e-29]; DNA polymerase alpha [*Plasmodium falciparum*, E value 1.8]	2.5 / 2.8	1.4	
yorL	unknown; similar to DNA polymerase III, alpha subunit, truncation [*Staphylococcus epidermidis* RP62A, E value 1e-141]	2.9 / 2.8	1.2	+
yorK	unknown; similar to unknown; similar to single-stranded-DNA specific exonuclease (RecJ) [*Staphylococcus epidermidis* RP62, E value 1e-54]	2.6 / 3.0	1.6	
yorH	unknown; similar to excinuclease ABC subunit A [*B. halodurans* C-125, E value 0.034]	3.0 / 2.2	1.4	+
yorG	unknown; similar to ATP/GTP binding protein [Bacteriophage SPBc2, E value 0.0]	4.1 / 3.8	1.1	+
yoqZ	unknown; similar to phage-related protein YmaC [*B. licheniformis* ATCC 14580, E value 2e-46]; DNA helicase *recQ*1 [*Aedes aegypti*, E value 1.3]	2.5 / 4.0	1.8	
ligB	DNA ligase (ATP-dependent)	5.4 / 7.0	1.5	
yoqR	unknown; similar to hypothetical protein BSU20540 [*B. subtilis* subsp. *subtilis* str. 168, E value 2e-62]	6.0 / 7.6	4.4	
yoqL	unknown; similar to HNH endonuclease: HNH nuclease [*Prochlorococcus marinus* str. AS9601, E value 1e-04]	4.1 / 3.8	2.4	
yoqC	unknown; similar to DNA polymerase III subunit delta [*Pseudoalteromonas haloplanktis* TAC125, E value 6.0]	2.6 / 2.8	2.0	+
yopY	unknown; similar to ATPase involved in DNA repair [*C. acetobutylicum* ATCC 824, E value 9.3]	3.4 / 5.0	1.4	+
yopW	unknown; similar to DNA gyrase subunit B [*Streptococcus downei*, E value 0.35]	2.5 / 5.4	2.2	
yopI	unknown; similar to N^6-adenine-specific DNA methyltransferase, D12 class [*Alkaliphilus metalliredigenes* QYMF, E value 1.5]	3.1 / 4.3	1.8	
yopC	unknown; similar to DNA polymerase, zeta catalytic subunit [*Entamoeba histolytica* HM-1:IMSS, E value 0.43]	2.5 / 2.1	1.4	
yonV	unknown; similar to CCAAT-box DNA binding protein subunit B [*Plasmodium yoelii yoelii* str. 17XNL, E value 0.001]; DNA repair protein [*Acanthamoeba polyphaga mimivirus*, E value 0.30]	4.0 / 3.7	1.9	
yonI	unknown; similar to DNA polymerase I [*Brachyspira hyodysenteriae*, E value 4.6]	3.0 / 2.7	1.4	
yonF	unknown; similar to prophage, terminase, ATPase subunit [*Staphylococcus epidermidis* RP62A phage SP-beta, E value 1e-132]	2.7 / 1.8	1.9	
yonE	unknown; similar to DNA replication licensing factor [*Theileria annulata* str. Ankara, E value 0.88]	2.6 / 3.3	1.8	
yonC	unknown; similar to thymidylate kinase (tmk) [*Methanocaldococcus jannaschii* DSM 2661, E value 7.7]	4.1 / 4.8	2.4	
yomK	unknown; similar to ATP-dependent exoDNAse (exonuclease V) beta subunit [*Francisella tularensis* subsp. *novicida* U112, E value 5.7]	2.5 / 2.7	1.7	

yomH	unknown; similar to DNA polymerase III subunits gamma and tau [*Helicobacter acinonychis* str. Sheeba, E value 4.4]	2.6 / 3.0	1.1		
yomG	unknown; similar to DNA double-strand break repair rad50 ATPase [*Methanococcus maripaludis* S2, 2e-04]; DNA repair protein (RecN) [*Streptococcus agalactiae* 2603V/R, E value 3e-04]	6.1 / 5.4	1.3		
yomF	unknown; similar to conserved hypothetical phage-related protein [*C. botulinum* phage C-St, E value 7e-15]	2.6 / 4.2	1.4		
yomE	unknown; similar to N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase [*Gallus gallus*, E value 2e-07]	6.2 / 5.3	2.2		
yomD	unknown; similar to excinuclease ABC, C subunit-like [*Polaromonas* sp. JS666, E value 0.008]; site-specific DNA endonuclease [*Pseudendoclonium akinetum*, E value 0.46]	3.0 / 3.4	1.7		
yolF	unknown; similar to phenylalanine tRNA synthetase like beta subunit [*Strongylocentrotus purpuratus*, E value 3.0]	2.5 / 2.9	1.8		
uvrX	ImpB/MucB/SamB family protein [Bacteriophage SPBc2]; UV-damage repair protein [*B. subtilis* subsp. *subtilis* str. 168 E value 0.0]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 1e-103]	4.7 / 7.1	1.1	+	
yolD	unknown; similar to putative chromosome segregation protein, SMC ATPase superfamily [*Prochlorococcus marinus* subsp. *pastoris* str. CCMP1986, E value 5.1]; cyclic nucleotide-binding domain containing protein [*Tetrahymena thermophila* SB210, E value 8.7]	2.7 / 3.0	1.5	+	
yolC	unknown; similar to ATP-dependent DNA helicase (RecG) [*Psychroflexus torquis* ATCC 700755, E value 0.60]	5.1 / 4.3	1.6	+	
yolA	unknown; similar to helicase (RecQ) [*Pasteurella multocida* subsp. *multocida* str. Pm70, E value 5.5]	3.0 / 4.7	1.1		
yokA	unknown; similar to resolvase, N-terminal: recombinase [*B. weihenstephanensis* KBAB4, E value 1e-40]	8.3 / 9.7	1.4		
yppQ	unknown; similar to methionine sulfoxide reductase B [*B. subtilis* subsp. *subtilis* str. 168, E value 4e-82]	4.0 / 4.2	3.6		
ndk	nucleoside diphosphate kinase	4.5 / 6.1	3.4		
hbs	non-specific DNA-binding protein HBsu	4.3 / 8.0	2.2	+	
yqkB	unknown; similar to molybdopterin oxidoreductase [*Alkalilimnicola ehrlichei* MLHE-1, E value 5.6]	2.7 / 3.1	1.9	+	
yqjZ	unknown; similar to antibiotic biosynthesis monooxygenase [*Pseudomonas fluorescens* PfO-1, E value 3e-31]	2.7 / 3.0	1.4	+	MMC, UV
yqjY	unknown; similar to acetyltransferase, GNAT family [*B. anthracis* str. Ames, E value 5e-40]	5.1 / 4.0	1.5	+	MMC, UV
yqjX	unknown; similar to similar to YolD homolog [*B.* sp. NRRL B-14911, E value 1e-04]	3.0 / 3.4	2.0	+	MMC, SA, UV
yqjW	unknown; similar to DNA polymerase IV 2 (Pol IV 2), ATP/GTP-binding protein (ImpB/MucB/SamB family) [*B. halodurans* C-125, E value 1e-142]; DNA-damage repair protein [*Oceanobacillus iheyensis* HTE831, E value 1e-81]	9.1 / 11.3	1.8	+	MMC, SA, UV

yqjH	unknown; similar to DNA polymerase IV [*B.* sp. NRRL B-14911, E value 1e-130]; nucleotidyltransferase / DNA polymerase involved in DNA repair [*B. anthracis* str. A2012, E value 7e-83]	3.5 / 5.8	2.5		
nusB	probable transcription termination	2.6 / 2.7	1.3		
recO	DNA repair and homologous recombination	3.3 / 7.0	1.5		MMC, UV
lepA	GTP-binding protein	4.1 / 3.8	2.4		
yqbQ	unknown; similar to DNA topoisomerase I [*C. difficile* 630, E value 1.4]	6.4 / 5.3	2.7		
yqaR	unknown; similar to exodeoxyribonuclease V, gamma chain [*Borrelia garinii* PBi, E value 0.077]; single-stranded nucleic acid binding R3H [*Thermosipho melanesiensis* BI429, E value 0.50]	2.7 / 3.3	1.5		
yqaM	unknown; similar to DNA replication protein, DnaC-like [*Acidobacteria bacterium*, Ellin345, E value 5e-14]	4.0 / 3.4	1.4		
yrkE	unknown; similar to FAD-dependent pyridine nucleotide-disulphide oxidoreductase [*C. cellulolyticum* H10, E value 5e-14]	2.5 / 2.6	1.8		
yraK	unknown; similar to oxidoreductase [*Bradyrhizobium* sp. BTAi1, E value 8e-14]; epoxide hydrolase [*Myxococcus xanthus* DK 1622, E value 2e-07]	4.1 / 3.0	3.7	+	
yrrK	unknown; similar to Holliday junction resolvase-like protein [*B. subtilis* subsp. *subtilis* str. 168, E value 8e-72]	5.7 / 12.4	1.9		
yrvE	unknown; similar to single-strand DNA-specific exonuclease (RecJ) [*B. licheniformis* ATCC 14580, E value 0.0]	4.7 / 6.3	2.1		
infC	initiation factor IF-3	2.7 / 1.9	1.5		
yuiA	unknown; similar to DnaJ-class molecular chaperone with C-terminal Zn finger domain [*Rubrivivax gelatinosus* PM1, E value 0.068]; phosphoesterase (RecJ-like) [uncultured methanogenic archaeon RC-I, E value 0.20]	5.0 / 4.1	1.7		PS
pucB	xanthine dehydrogenase (purine degradation)	4.0 / 6.4	2.9		
gerAA	germination response to L-alanine	3.1 / 5.4	3.8		
yvrE	unknown; similar to senescence marker protein-30 family protein [*B. cereus* G9241, E value 4e-64]	2.8 / 3.0	2.6		GS, HS
tpiA	triose phosphate isomerase	2.7 / 2.0	2.5		
trxB	thioredoxin reductase	2.8 / 3.3	1.7		
yvoD	unknown; similar to nucleoside recognition [*Rubrobacter xylanophilus* DSM 9941, E value 1e-74]	4.1 / 5.7	2.8		
tagC	possibly involved in polyglycerol phosphate teichoic acid biosynthesis (DinR-binding site; DNA-damage inducible; *dinC*)	6.4 / 9.0	1.4	+	MMC, PS, UV
ywqH	unknown; similar to single-strand DNA-binding protein [*B. licheniformis* ATCC 14580, E value 2e-37]	7.1 / 10.8	2.6		
atpG	ATP synthase (subunit gamma)	2.6 / 3.0	1.8		
atpA	ATP synthase (subunit alpha)	2.5 / 2.7	2.1		
pyrG	CTP synthetase	3.3 / 2.6	1.7		
ywhD	unknown; similar to conserved hypothetical protein [*B. cereus* E33L, E value 1e-72]; DNA primase [*Buchnera aphidicola* str. APS (*Acyrthosiphon pisum*), E value 9.1]	4.0 / 3.1	3.7		
ywcE	unknown; similar to spore germination protein [*C. thermocellum* ATCC 27405, E value 4.3]	2.7 / 4.6	2.6		

yxkI	unknown; similar to heat shock protein HtpX [*B. subtilis* subsp. *subtilis* str. 168, E value 1e-07]	2.6 / 2.4	3.0	
nupC	pyrimidine-nucleoside transport protein	3.0 / 4.7	2.1	
dnaC	replicative DNA helicase	2.7 / 3.5	1.6	
ssb	single-strand DNA-binding protein	11.5 / 17.3	3.4	MMC, UV
yyaF	unknown; similar to GTP-dependent nucleic acid-binding protein EngD [*B. licheniformis* ATCC 14580, E value 1e-177]	5.6 / 6.4	1.5	

[a] For details on the data presentation, see Table 3-8, footnotes [a] through [e].

To clarify the functional role of the up-regulated genes (Table 3-22) after high vacuum exposure and 60 min germination, seven GO groups were identified and listed in Table 3-23 and the most prominent functional categories in relation to their gene expression are discussed, as described in 3.2.3, 3.3.3 and 3.4.4.

Table 3-23 Highly induced genes after (30^1 and 450^2 d) high vacuum exposure and their functional affiliation in 7 GO groups[a]

GO 1. DNA repair and modification/restriction

wild-type[1]: *adaB, dinB, lexA, tagC (dinC), uvrX, yacK (disA), ydiO, yeeD•, yirY, yoeB•, yomD•, yomG, yonV•, yopI•, yopY•, yorH•, yozK, yqjH, yqjW*

wild-type[2]: *adaB, dinB, lexA, tagC (dinC), uvrX, yacK (disA), ydiO, yeeD•, yirY, yoeB•, yomD•, yomG, yonV•, yopI•, yopY•, yozK, yqjH, yqjW*

recA[1]: *yirY, yqjH*

GO 2. DNA recombination

wild-type[1]: *recA, recF, recO, yefB, yefC, yloC, yneB, yocI, yokA, yolA•, yolC•, yomK•, yorK, yozJ•, yrrK, yrvE*

wild-type[2]: *recA, recF, recO, yefB, yefC, yloC, yneB, yocI, yokA, yolA•, yolC•, yomK•, yorK, yrrK, yrvE*

recA[1]: -

GO 3. DNA replication

wild-type[1]: *dnaC, dnaX, ligA, ligB, ssb, ykuL•, ymzC•, yomH•, yonE•, yonI•, yopC•, yorL, yorS•, yosH•, yosL•, yqaM, ywhD•, ywqH*

wild-type[2]: *dnaC, dnaX, ligA, ligB, ssb, ykuL•, ymzC•, yomH•, yonE•, yonI•, yorL, yorS•, yosH•, yosL•, yqaM, ywhD•, ywqH*

recA[1]: *ssb, ywhD•, ywqH*

GO 4. DNA packaging and segregation

wild-type[1]: *hbs, ydcP•, yolD•, yopW•, yoqC•, yozL•, yqbQ•, yqjX*

wild-type[2]: *hbs, ydcP•, yolD•, yopW•, yoqC•, yozL•, yqbQ•, yqjX*

recA[1]: *yqbQ•*

GO 5. Nucleotides, nucleobases, nucleotide binding and nucleic acid metabolism

wild-type[1]: *gmk, lepA, ndk, pucB, purR, pyrG, pyrH, yhjM, yonC•, yoqL•, yorG, yqaR•, yrkE, yvoD, yyaF*

wild-type[2]: *adk, gmk, lepA, ndk, pucB, pyrG, yhjM, yonC•, yoqL•, yorG, yqaR•, yrkE, yvoD, yyaF*

recA[1]: *ndk, pucB, yvoD*

GO 6. Adaptation, detoxification and response to stress (high vacuum/extreme dryness stimulus)

wild-type[1]: *clpY, ycbR, yhjJ, yppQ, yqjZ, yqkB•, yraK, yuiA•, yxkI*

wild-type[2]: *clpY, ycbR, yhjJ, yppQ, yqjZ, yqkB•, yraK, yuiA•*

recA[1]: *yppQ, yraK, yxkI*

GO 7. Other functions

GO 7.1 Germination and cell envelope

wild-type[1]: *gerAA, gerPF, murG, nupC, yhjL, ywcE•*

wild-type[2]: *gerAA, gerPF, murG, nupC, yhjL, ywcE•*

recA[1]: *gerAA, ywcE•*

GO 7.2 Bioenergetics

wild-type[1]: *atpA, atpG, trxB*

wild-type[2]: *atpA, atpG, trxB*

recA[1]: -

GO 7.3 Other functions, or/and unknown and hypothetical proteins

wild-type[1]: *cdsA, ecsB, infC, nusB, rplX, sigW, thiL, tpiA, tsf, ybcS, ycbN, yhjK, yjcG, ykkC, yloV, , yolF•, yomE, yomF, yonF, yoqR, yoqZ, yotB, yqjY, yvrE*

wild-type[2]: *cdsA, ecsB, nusB, rplX, sigW, thiL, ybcS, ycbN, yjcG, yloV, , yolF•, yomE, yomF, yoqR, yoqZ, yotB, yqjY, yvrE*

recA[1]: *tpiA, yhjK, yoqR, yvrE*

• Gene with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). For details on the gene function, see Table 3-22. [a] For details on the data presentation, see Table 3-9.

Twenty of the 119 ≥ 2.5-fold up-regulated genes (16.8 %) were directly assigned to DNA repair, modification and restriction, as can see in GO 1. (Table 3-23). Mainly, DNA-damage inducible genes e.g. *dinB* (nuclease inhibitor), *tagC* (former *dinC*) and four type IV DinB-like DNA polymerase (*uvrX*, *yqjH*, *yqjW* and *yozK*) were significantly induced. Furthermore, two DNA-methyltransferase, *adaB* (a O^6-methylguanine-DNA methyltransferase, involved in base excision repair (Martins-Pinheiro et al., 2007)) and the predicted *Rhodopseudomonas palustris* BisB18-like cytosine-specific DNA-methyltransferase *ydiO* as well as the putative DNA scanning protein *yacK* (*disA*) were found to be highly responsive to vacuum exposure. Three *rec*-genes, *recA* (multifunctional protein involved in homologous recombination and recombination-mediated repair), *recF* (DNA repair and genetic recombination; with ssDNA and dsDNA strand break binding affinity) and *recO* (protein involved in the dsDNA interaction of *recF and recR* during homologous recombination/recombination-mediated repair), five putative recombinase/resolvase-like protein (*yefB*, *yefC*, *yneB*, *yokA* and *yrrK*) and seven probable DNA helicases (*ycbR*, *yloC*, *yozJ*, *yocI*, *yoqZ*, *yolA* and *dnaC*) were found to be highly activated and implicated in their function in DNA recombination and replication processes. Gene function and similarities to other bacteria can be seen in Table 3-22.

In addition, three single-strand binding proteins (*hbs*, *ssb* and a *B. licheniformis* ATCC 14580-like single-strand DNA-binding protein (*ywqH*)) were up-regulated in vacuum-exposed spores after 60 min germination. All three ssDNA-binding encoding genes and both *lig*-genes encoding DNA ligases (*ligA* and *ligB*) were also found to be induced after X-ray and accelerated heavy ions irradiation leading to assumption that both treatments induced significant numbers of single- and double-strand DNA breaks, which have to be repaired to ensure genome integrity (GO 2. and 3. in Table 3-23).

Nine type III DNA polymerases (*dnaX*, *ymzC*, *yosL*, *yosH*, *yorS*, *yorL*, *yoqC*, *yopC* and *yomH*), two type I DNA polymerases (*ykuL* and *yonI*) as well as sixteen genes involved in nucleotide new synthesis were induced. This indicates that DNA damage induced by high vacuum requires restoration of genomic stability via new DNA replication (GO 4. and GO 5. in Table 3-23).

Further on, nine genes involved in the stress response e.g. *yhjJ* (a putative oxidoreductase, similar to*B. thuringiensis* str. Al Hakam), *yppQ* (a previously mentioned sulfoxide reductase, see 3.4.4), *yraK* (a

predicted oxidoreductase (a *Bradyrhizobium* sp. BTAi1 homolog) or epoxide hydrolase (a *Myxococcus xanthus* DK 1622-like enzyme)) and *yxkI* (a probable heat shock protein) were up-regulated.

Four known (*gerAA*, *gerPF*, *murG* and *nupC*) and two "*y*"-genes (*yhjL* - a *Thermoanaerobacter tengcongensis* MB4-like putative regulator of cell wall biogenesis and *ywcE* - a predicted spore germination protein, similar to *C. thermocellum* ATCC 27405), involved in germination and cell envelope processes, were found to be triggered by vacuum-exposure of the spores during spore germination (for details see GO 6., Table 3-23), similar observations were obtained after UV or ionizing irradiation of spores (see 3.2.3, 3.3.3 and 3.4.4).

After 30 d exposure to extreme dryness achieved by high vacuum exposure *recA*, *ssb* (ssDNA-binding), *yefB* (a resolvase/recombinase), *yqjW* (a DinB-like polymerase) and *yokA* (N-terminal resolvase/recombinase) were the five highest up-regulated wild-type genes, whereas in *recA*-deficient spores the following five genes were strongly induced: *yoqR* (an unknown hypothetical protein), *yirY* (a *Symbiobacterium thermophilum* IAM 14863-like DNA repair exonuclease), *yraK*, *gerAA* and *ywhD* (an unknown hypothetical protein). After 1 year and 3 months (450 d) extreme desiccation *recA*, *ssb*, *yirY*, *yrrK* (a predicted Holliday junction resolvase-like protein, similar to*B. subtilis* subsp. *subtilis* str. 168) and *yneB* were the five most highly induced genes in the wild-type strain.

In order to get further confirmation of the validity of the DNA microarray data of both putative site-specific recombinases (members of resolvase family; *yefB* similar to *Geobacillus kaustophilus* HTA426 and *yefC* a *B. cereus* G9241-like homolog) they were selected for RT-PCR experiments (2.4.7). Fig. 3-27 shows the DNA bands of the amplified cDNA after RT-PCR (as described in 2.4.7). The amplified cDNA band for the estimated RT-PCR product was in the expected product size (264 bp).

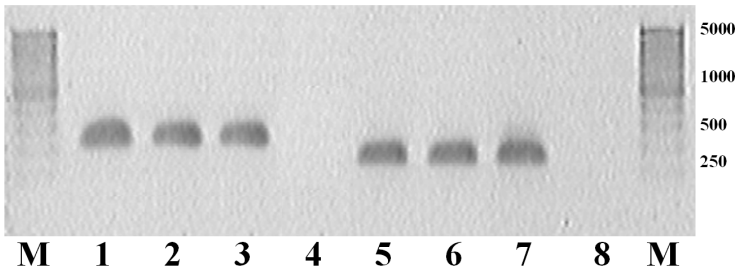


Fig. 3-27 RT-PCR analysis of *yefB* (1-4) and *yefC* (5-8) - transcripts of wild-type *B. subtilis* 168 spores after vacuum treatment and 60 min germination. Rows loaded with *yefB*: (1) 30 d vacuum, (2) 450 d vacuum, (3) X-ray irradiated, (4) non-vacuum exposed control: and rows loaded with *yefC*: (5) 30 d vacuum, (6) 450 d vacuum, (7) X-ray irradiated, (8) non-vacuum exposed control, and (M) standard DNA marker (Hyper Ladder I, Bioline GmbH, Luckenwalde, Germany).

Similar cDNA band pattern were obtained for *yqjH*, *yneB* and *yobH* from RNA samples isolated from spores exposed to high vacuum after 60 min germination (detailed gene functional characterization and ontology can be seen in Table 3-22 and 3-23).

3.6 Experimental studies of meteorite impact ejection processes of microorganisms

The aim of this study is to provide an experimental basis for the discussion of "*Lithopanspermia*", the transport of microorganisms between planets, e.g., Mars and Earth, by means of meteorites (Horneck et al., 2001b; 2007; Stöffler et al., 2007). In precise shock wave recovery experiments bacterial endospores (in this thesis), epilithic and cryptoendolithic microbial associations (in cooperation with Prof. Dr. C. S. Cockell and Dr. J.-P. P. de Vera) were analyzed on their survivability to applied shock pressures in a range from 5 to 50 GPa which is the range of pressures experienced by the known Martian meteorites. To gain more realistic settings of the Martian surface conditions adequate pre-shock temperatures and different rock compositions were tested. The actual shock pressure of the recovered samples was measured in cooperation with the Mineralogy-research group leaded by Prof. Dr. D. Stöffler (Humboldt-University of Berlin, Germany). This work was performed within the projects (HO 1508/3-1 to G. Horneck; STO 101/39-1/2 to D. Stöffler; RA 1049/1-2 to E. Rabbow) of the DFG-Priority Program "Mars and terrestrial planets" (SPP 1115).

3.6.1 Survival of *B. subtilis* spores after exposure to high shock pressures

Spores in a dry layer mounted between gabbro discs were exposed to shock pressures in a range from 5 to 50 GPa, a pressure range known from Martian meteorites found on Earth (Fritz et al., 2005). The

actual shock pressure of the recovered samples was determined by refractive index measurements of the host minerals quartz and plagioclase for which accurate calibration data are available (in cooperation with Prof. Dr. D. Stöffler and Dipl. chem. mineral. C. Meyer). After exposure to different shock pressure scenarios, spores were stripped off the gabbro discs by repetitive PVA technique (as described in detail in 2.2.2.1) resulting in > 95 % recovery of the spores. Survival of the spores was determined from their ability to form colonies, as described in detail in 2.1.5. Surviving fractions were determined as N/N_0 where N was the number of colony forming units (CFU) of the treated samples and N_0 that of the untreated control (as described in detail in 2.5.1). Mean values and standard errors were calculated for each sample from up to six replicate plates. Survival of shocked spores of *B. subtilis* decreased exponentially with increasing shock pressure (Fig. 3-28). The regression factor (Pearson correlation coefficient) of a second order regression amounted to r = 0.989. The survival rate was reduced to about 10^{-4} for spores exposed to a shock pressure of 41.5 GPa. At higher shock pressures, such as 50 GPa, survival rates dropped below their threshold of detection at 5×10^{-8}. The "detection threshold" depends on the initial spore number mounted on the rock material, which was 1×10^{8} in this work. A marker test of the colonies from the experiment samples (Horneck et al., 2001a; 2001b; 2007) showed that they were free of any contamination.

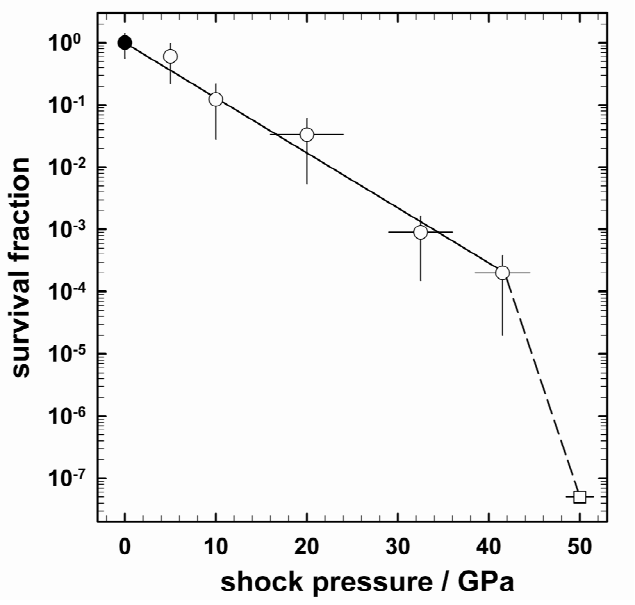


Fig. 3-28 Shock pressure-survival curve of spores of *his*-, *met*- and *leu*-auxotrophic *B. subtilis* TKJ 6312 (open circles) encased in gabbro plates plotted against the induced shock pressures. The open square indicate survival below the threshold of detection. At a shock pressure of 50 GPa, survival rates dropped below the threshold of detection of 10^{-8} (the open square indicates no survival at 50 GPa). The "detection threshold" for the survival rate is defined by the initial microbe population in the rock material. Data are averages +/- standard deviation.

In Table 3-24 the key parameters of the spore survival after high shock-pressure exposure are given (according to 2.5.1, Table 2-6). It shows that 10 % of the spores survived exposure to 12.1 GPa - a value that is at the lower pressure range of Martian meteorites (Fritz et al., 2005). It should be noted that the shock pressure-survival curve in Fig. 3-28 was obtained at pre-shock room temperature. This curve was taken as reference when using different pre-shock temperatures.

Table 3-24 Survival characteristics of the shock-pressure treated *B. subtilis* spores encased in gabbro

Curve characteristics	*B. subtilis* 168 TKJ 6312 (*splB uvrB*)
ShP_{10} (GPa)	12.1 ± 4.3
IC (GPa^{-1})	$(2.13 \pm 0.24) \times 10^{-1}$
n	1.32 ± 0.16
R^2 / *r*	0.989

ShP = characteristic shock pressure.

To obtain more realistic settings of Martian environmental conditions, three different pre-shock temperatures (i.e. 193, 233 or 293 K), which are more adequate to the early or present Martian surface data, three different types of rock material (i.e. gabbro, sandstone or dunite) and two different types of Martian soil analogues (i.e. Martian regolith MRS07 or artificial halite) were used in shock recovery experiments with spores. Details of the soil and rock composition are given in chapter 2.2 (for details see 2.2.6.1, Fig. 2-4). In Fig. 3-29 the survival of *B. subtilis* spores is shown at different shock conditions with regard to pre-shock temperature, water content, and/or Martian-like soil/rock analogue). In those experiments, a Rif^r mutant of *B. subtilis* 168 was used because of its wild-type DNA, which allowed to compare its repair capability to that of the TKJ6321 strain.

It is remarkable that the survival of spores shocked in gabbro at a pre-shock temperature of 193 K was about 5- to 10-times higher than that of spores shocked at pre-shock room temperature (293 K). Even at shock pressures of 50 GPa, where no survival was detectable for pre-shock room temperature (293 K) (Fig. 3-28), survival was about 10^{-4} for spores encased in gabbro when shocked. Because calculations have shown that the content of water essentially influences the peak-shock temperature during these experiments (Horneck et al., 2007) shock recovery experiments were performed with air-dried as well

as with moisturized spores, which were distributed inside of sandstone by soaking of a spores suspension Fig. 3-29 shows that air-dried samples survived shock pressures at 30 GPa significantly better than the moisture spores. It was generally observed that spores shocked at pre-shock temperatures of 233 K or 193 K, i.e. initially cooled spores, survived significantly better than their analogous spores shocked at a pre-shock temperature of 293 K (for detail of the spore survival see Table 3-25). Spores, embedded in halite or mixed with MRS07, were inactivated by roughly four orders of magnitude, which is the similar survival capability compared with spores dried on gabbro and shocked at 30 GPa room temperature (Table 3-25). One major reason for this similar spore survival for the halite and MRS07 sample is the applied high compression of the spore-powder mixture during the sample assembly, which led to roughly the same compaction as of gabbro and lower porosity compared to the initial spore-powder composition. Further on, spores dried on dunite survived about 3-times better than spores on gabbro a shock pressure of 40 GPa at 293 K. This could be explained due the different responsivity of gabbro and dunite at the same shock pressure, i.e. mainly through the lower post-shock temperature formation in dunite compared to gabbro, which favor a high spore survival.

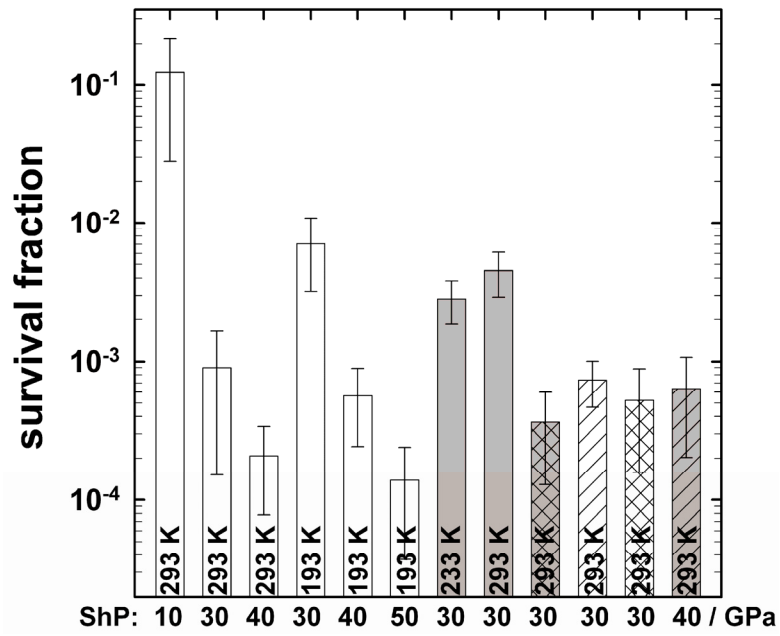


Fig. 3-29 Shock pressure-survival correlation of spores of *B. subtilis* RM01 (carrying a mutation in *rpoB* Q469L; leading to an increased rifampicin-resistance) immobilized on gabbro (white column), dry sandstone (grey column), moisturized sandstone (grey meshed column), halite (white fasciated column), Martian regolith MRS07 (white meshed column) and dunite (grey fasciated column). Initial temperature and applied shocked pressure (ShP) are stated in the figure. Data are averages +/- standard deviation.

In Table 3-25 the individual numbers of spore survivors and their degree of inactivation are shown, depending on the initial *B. subtilis* spores concentration (about 1×10^8/run depending on shock substrate).

Table 3-25 Shock-pressure survival of Martian conditions adapted *B. subtilis* spores

Experimental conditions	Surviving spore fraction / inactivation
gabbro, 10 GPa, 293 K	18.450.000 / 1.2×10^{-1}
gabbro, 30 GPa, 293 K	135.300 / 9.0×10^{-4}
gabbro, 40 GPa, 293 K	31.200 / 2.1×10^{-4}
gabbro, 30 GPa, 193 K	1.056.000 / 7.0×10^{-3}
gabbro, 40 GPa, 193 K	85.000 / 5.7×10^{-4}
gabbro, 50 GPa, 193 K	20.800 / 1.4×10^{-4}
sandstone dry, 30 GPa, 233 K	424.700 / 2.8×10^{-3}
sandstone dry, 30 GPa, 293 K	678.500 / 4.5×10^{-3}
sandstone wet, 30 GPa, 293 K	54.500 / 3.6×10^{-4}
halite, 30 GPa, 293 K	110.100 / 7.3×10^{-4}
MRS07, 30 GPa, 293 K	78.200 / 5.2×10^{-4}
dunite, 40 GPa, 293 K	95.300 / 6.4×10^{-4}

3.6.2 Shock pressure-induced mutagenesis on the colony morphology of *B. subtilis*

Mutagenesis in stressed microorganisms inidicates that the stressor has induced damage to the DNA. To find out whether the shock pressure treatment was mutagenic, the colonies of the shock pressure-survivors were screened for sporulation defective (Spo^-) mutants. Colonies of *B. subtilis* 168 Spo^+ strains are opaque and brown pigmented, while Spo^- mutants are translucent and/or remain unpigmented after incubation on sporulation medium (Fajardo-Cavazos et al., 2005).

To search for Spo^- mutants in shock-pressure stressed spores it was first necessary to confirm that the samples were free of contaminants. As genetic markers the *B. subtilis* 168 strain RM01 used carries a Rif^r mutation (mutation Q469L in *rpoB*, isolated in this work, see 2.3.3) and a tryptophan auxotrophy

marker. In a first step the shock pressure-stresssed spores were recovered from simulated meteorite material (i.e. gabbro) and analyzed for their vitality on NB agar plates (Fig. 3-28, Table 3-25).

5×10^3 ("survivor") spores (from gabbro, stressed at 30 or 40 GPa at a pre-shock temperature of 193 K) were then plated on SSM-medium and incubated at optimal growth and sporulation conditions (see 2.1.3 and 2.1.4). It was noted by visual inspection of the colonies that the colonies were consistent with *B. subtilis* 168 morphology and visually indistinguishable from strain *B. subtilis* 168. To determine the phenotype of the spores, a total of 100 colonies each from the 30 GPa (Fig. 3-30B) and the 40 (Fig. 3-30C) shock pressure-treated samples as well as the un-treated control group (Fig. 3-30A) were picked onto SMM-Rif (50 µg of rifamicin per ml) and tryptophan-marker test (according to Horneck et al., 2001a; 2001b; 2007) the strain needs at least 50 µg tryptophan per ml of minimal media to grow). All colonies were prototrophic (Rif-resistant and *trp*-auxotroph), lending further support to the notion that all colonies were genetically identical to *B. subtilis* 168 (strain RM01) and not environmental contaminants.

Colonies arising from the non-treated control (Fig 3-30A) appeared homogeneous and indistinguishable from *B. subtilis* 168 (RM01). In contrast, a few spore survivors from 30 and 40 GPa formed colonies that resembled *B. subtilis* 168 (RM01) but exhibited decreased pigmentation (Hullo et al., 2001) up to total bleach effect of the visible macrocolony, which is characteristic of sporulation-defective (Spo^-) *B. subtilis* mutants (Piggot and Coote, 1976).

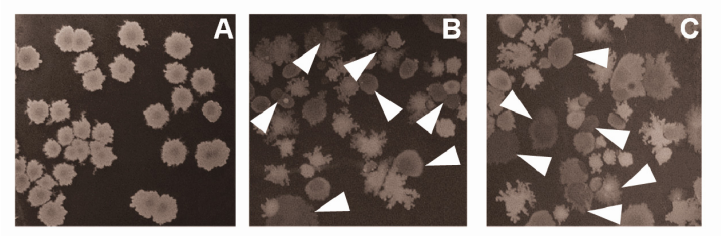


Fig. 3-30 Morphology of colonies arising on SSM-medium (2.1) from *B. subtilis* spores plated from non-shocked control sample (**A**), gabbro-shocked samples **B** (30 GPa, 193 K) and **C** (40 GPa, 193 K). Arrowheads indicate Spo^- mutants.

Further examination of 247/281 colonies from 30/40 GPa sample revealed 19/26 Spo^- colonies (7.7 % / 9.3 %), whereas examination of 305 colonies from non-shocked control revealed only two Spo^- colonies (0.006 %). From the above observations it was apparent that mutations leading to the Spo^-

phenotype had been induced by the applied shock pressure. Similar observations on induced Spo$^-$ mutagenesis were reported by Fajardo-Cavazos et al. (2005) on recovered vital *B. subtilis* spores, located on artificial meterorite material, after a hypervelocity atmospheric (re-)entry. The treatment, namely a simulated meteorite impact and ejaction process includes exposure to the following possibly mutagenic physical parameters: (i) extreme shock pressure (30/40 GPa), (ii) peak-pressure and post-pressure heating, especially of the impact-ARMCO iron container, accommodating the rock with the encased *B. subtilis* spores and (iii) the impact of mechanical stress by friction and/or crushing, alone or in combination with the other factors.

It has been shown that *B. subtilis* spores exposed to extreme low pressure in low Earth orbit accumulated mutagenic and lethal levels of DNA damage in dormant spores (reviewed in Nicholson et al., 2000). Exposure to dry heat (45 min, 120°C) has been observed to raise the mutation frequency to nalidixic acid resistance in the *gyrA* gene of *B. subtilis* spores by two orders of magnitude (del Carmen Huesca-Espitia et al., 2002); however, in this work the high pressure treated samples were exposed to heat for less than fractions of a min only (in Table 2-5 analogous temperature data for shock-treated spores at 293 K pre-shock temperature are shown).

3.7 Functional characterization of the *ykoWVU*-operon in spore resistance

Recently, Weller et al. (2002) identified in *B. subtilis* a Ku homolog (encoded by the *ykoV* gene), which retains the biochemical characteristics of the eukaryotic Ku heterodimer. The bacterial Ku specifically recruits a DNA ligase (encoded by *ykoU*) to DNA ends and thereby stimulates DNA ligation. Loss of these proteins leads to hypersensitivity of stationary-phase *B. subtilis* cells to ionizing radiation (Weller et al., 2002). From the observation that the Ku system is conserved in spore-forming bacterial species (e.g., *Bacillus* and *Streptomyces* spp.), Weller et al. (2002) speculated that NHEJ via the prokaryotic Ku system might function during subsequent spore germination to repair DSB induced in dormant bacterial spores (Bowater and Doherty, 2006; Wang et al., 2006).

3.7.1 Role of the *ykoWVU*-operon in the spore resistance to ionizing radiation

For studying the effects of ionizing radiation-induced DNA damage on spore survival and the imprtance of the *ykoWVU*-operon in these processes, X-ray dose-survival curves of spores with

mutations in the *ykoWVU*-operon (NHEJ-deficient spores) were compared with those of wild-type, *splB*, and *recA* strains (Fig. 3-31).

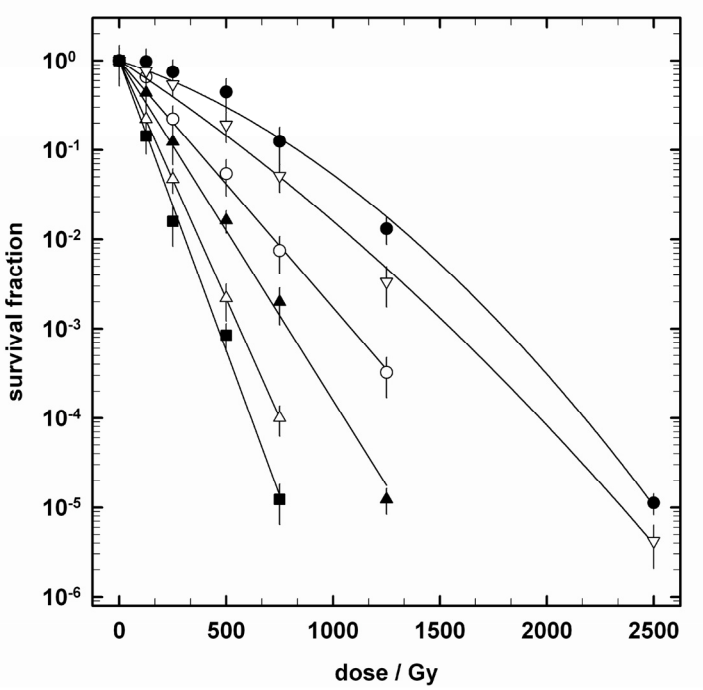


Fig. 3-31 Dose-survival curve of *B. subtilis* 168 spores after irradiation with X-rays: wild-type (solid circles), *recA* (open circles), *ykoU* (solid triangles up), *ykoV* (open triangles up), *ykoU ykoV* (solid squares) and *splB* (open triangles down). Data are averages +/- standard deviation (n = 3).

It is assumed that DNA double-strand breaks (DSB), which are the most critical damage caused by ionizing radiation (Micke et al., 1994; Weller et al., 2002) or desiccation (Potts, 1994) in vegetative cells, are also induced in bacterial spores (Dose et al. 1991; 1992). There are two alternative routes of ionizing radiation damage to the biological key components, such as proteins, RNA and DNA: either by direct energy absorption (direct radiation effect), or via interactions with radicals, e.g., produced by radiolysis of cellular water molecules (indirect radiation effect) (Hutchinson, 1985). Both processes mainly cause SSB or DSB in DNA (Dose et al., 1991; 1992; Nicastro et al., 2002). The decreased water content of the spore core, which may reduce the indirect effect, as well as the protection of the DNA by α/β type-SASP, has been suggested to be responsible for increased spore resistance to ionizing radiation (Setlow, 1995). However, so far, no DNA repair system has been identified in spores that act specifically on DNA damage caused by ionizing radiation. Therefore, wild-type and mutant spores were assayed for their resistance to ionizing radiation delivered in the form of X-rays (Fig. 3-31). After exposure to X-rays, strictly exponential survival curves were obtained for spores of all repair-deficient

strains of *B. subtilis* tested, while the wild-strain showed a slight shoulder in its survival curve (Fig. 3-31, with 5 times higher doses than in Fig. 3-16). In the case of X-rays resistance, the spores of *recA*, *ykoU*, *ykoV* and *ykoU ykoV* deficient strains were remarkably more sensitive than wild-type spores; by up to a factor of 7.9 with regard to the double mutant *ykoU ykoV*, which was again the most sensitive spore representative, as deduced from the D_{10} values (i.e., dose reducing survival to 10 %) (Table 3-26). These differences in sensitivity of the spores to X-rays showed a similar tendency as those reported for vegetative cells of the *B. subtilis* mutants (Weller et al., 2002). However, Weller et al. (2002) reported that in stationary-phase *B. subtilis* cells, *ykoU* mutants were more X-ray sensitive than *ykoV* mutants; interestingly, in spores the opposite was observed (Fig. 3-31, Table 3-26).

Table 3-26 Survival curve characteristics of NHEJ-deficient spores after X-ray irradiation

X-rays (150 keV/19 mA)	D_{10} (Gy)	D_0 (Gy)	D_q (Gy)	IC (Gy^{-1})
B. subtilis (wild-type)	971.8 ± 100.2	467.2 ± 55.2	66.3 ± 13.3	$(2.67 \pm 0.43)\times10^{-3}$
recA	343.2 ± 47.1	165.2 ± 40.3	n.d.	$(6.82 \pm 0.78)\times10^{-3}$
ykoU	274.1 ± 35.3	132.9 ± 25.0	n.d.	$(9.03 \pm 1.09)\times10^{-3}$
ykoV	188.8 ± 37.1	79.4 ± 18.0	n.d.	$(1.23 \pm 0.19)\times10^{-2}$
ykoU ykoV	149.4 ± 29.2	59.8 ± 17.6	n.d.	$(1.48 \pm 0.41)\times10^{-2}$
splB	720.4 ± 107.2	349.1 ± 25.3	32.4 ± 9.8	$(3.42 \pm 0.55)\times10^{-3}$

X-rays (150 keV/19 mA)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.56 ± 0.27	1	0.977	
recA	0.90 ± 0.10	0.35 ± 0.09	0.971	0.047
ykoU	0.85 ± 0.07	0.26 ± 0.06	0.996	0.042
ykoV	0.81 ± 0.05	0.19 ± 0.04	0.993	0.038
ykoU ykoV	0.75 ± 0.09	0.15 ± 0.08	0.991	0.020
splB	1.35 ± 0.16	0.75 ± 0.17	0.982	0.204

n.d. not determinable. The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). *P* = statistical significance of difference of data of respective strain compared to *B. subtilis* wild-type strain 168; $P \leq 0.05$ = significant difference. Data are averages and standard deviations ($n = 4$).

3.7.2 Role of the *ykoWVU*-operon in the spore resistance to extreme dryness

Spores are clearly much more resistant than their vegetative counterparts to extended desiccation both at atmospheric pressure and *in vacuo* (Nicholson et al., 2000). When typical laboratory vacuum systems (2.2.5) are often used for simulated extreme desiccation, wild-type spores often exhibit no detectable killing (as can seen in 3.5.1; Dose et al., 1991; 1992). A major reason for spore resistance to these processes is protection of spore DNA by SASP. SASP-deficient spores are much more sensitive to extended desiccation, and killing by these processes is accompanied by mutagenesis and DNA damage e.g. single-strand breaks and DNA-protein cross-links (Dose and Gill, 1995; Setlow, 1995). Previous studies of spores exposed to extreme desiccation via high vacuum indicated that wild-type spores were quite resistant while DNA repair-deficient spores showed significant sensitivity to extended dryness via vacuum (reviewed in Nicholson et al., 2000).

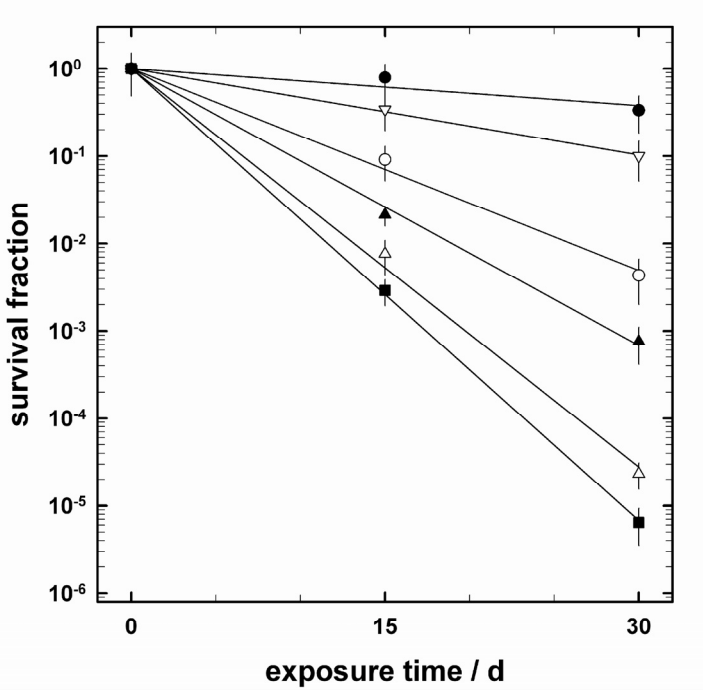


Fig. 3-32 Survival curve of *B. subtilis* 168 wild-type (solid circles), *recA* (open circles), *ykoU* (solid triangles up), *ykoV* (open triangles up), *ykoU ykoV* (solid squares) and *splB* (open triangles down) spores after high vacuum exposure (10^{-7} Pa) for 30 d. Data are reported as averages and standard deviations (n = 3).

Although significant lethality of wild-type spores in vacuo was not observed for short exposures of a few days, the survivors exhibited significant mutagenesis (Munakata et al., 1997; Fig. 3-25), indicating that DNA was definitely a target. Spores of the same strains as used in 3.7.1 were exposed to high vacuum (10^{-7} Pa) for up to 30 days and assayed for survival. Strict exponential inactivation kinetics

were observed for all strains (Fig. 3-32). After 30 days of vacuum-induced extreme desiccation, both wild-type and *splB* spores both showed a high level of survival (33.4 % and 19.8 %, respectively). In sharp contrast, spores carrying the *recA*, *ykoU, ykoV,* or *ykoU ykoV* mutations were dramatically more sensitive to high vacuum (Fig. 3-32), ranging from 77-fold (*recA* spores) to > 16500-fold (*ykoU ykoV* spores) as measured at 30 days (Table 3-27).

Table 3-27 Survival curve characteristics of NHEJ-deficient spores after high vacuum exposure

High vacuum (10^{-7} Pa)	IC	n	RF	R^2	*P*
B. subtilis (wild-type)	$(3.65 \pm 0.38)\times10^{-2}$	1.11 ± 0.06	1	0.974	
recA	$(1.81 \pm 0.44)\times10^{-1}$	1.09 ± 0.04	0.20 ± 0.06	0.995	0.036
ykoU	$(2.39 \pm 0.31)\times10^{-1}$	0.92 ± 0.09	0.15 ± 0.03	0.998	0.022
ykoV	$(3.56 \pm 0.40)\times10^{-1}$	1.06 ± 0.12	0.11 ± 0.04	0.990	0.018
ykoU ykoV	$(3.98 \pm 0.33)\times10^{-1}$	1.05 ± 0.08	0.09 ± 0.02	0.996	0.013
splB	$(7.68 \pm 1.05)\times10^{-2}$	1.05 ± 0.12	0.48 ± 0.13	0.990	0.048

The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). *P* = statistical significance of difference of data of respective strain compared to *B. subtilis* wild-type strain 168; $P \leq 0.05$ = significant difference. Data are expressed as averages and standard deviations ($n = 3$).

The extreme sensitivity of *ykoU ykoV*-deficient spores to high vacuum-induced desiccation is entirely consistent with the observation that exposure to high vacuum induces SSB and DSB formation in DNA (Dose et al., 1991; 1992; Nicastro et al., 2002). Thus, NHEJ is an extremely important determinant of spore survival to the high vacuum prevailing in space, a critical prerequisite for interplanetary transport of spores by natural processes or human spaceflight activities (Nicholson et al., 2000; 2005).

3.7.3 Role of the *ykoWVU*-operon in the spore resistance to UV radiation

Inactivation kinetics of wild-type and mutant *B. subtilis* spores were determined in response to mono- and polychromatic UV radiation spanning wavelengths from 254 - 400 nm (2.2.3.1 and 2.2.3.2; Fig. 3-33A-C). Nearly exponential UV survival curves were obtained for spores of all strains of *B. subtilis* tested (Fig. 3-33) and the best-fit curves were used to calculate F_{10} values (i.e. fluences reducing survival to 10 %) for statistical comparison (Table 3-28). In response to UV-C, examination

of the F_{10}-values showed that the *recA, ykoU*, *ykoV* and *ykoU ykoV* mutants produced spores with slight (1.5- to 2-fold) but significantly lower F_{10}-values than that of the wild-type strain (Table 3-28). In contrast, spores carrying the *splB* mutation were 3-fold more UV-C sensitive than wild-type (Table 3-28). The slight but significant UV sensitivity of *ykoU* and *ykoV* mutant spores likely reflects the fact that the majority of the 254-nm UV photoproducts in spore DNA are SP thymine dimers, whereas strand breaks such as SSB and DSB are only minor photoproducts (Slieman and Nicholson, 2000; 2001). Comparing the data to those obtained for X-rays it is remarkable that the observed differences in UV-C resistance of the mutant *versus* wild-type spores, determined as the repair capacity, were much less pronounced for UV-C radiation than those observed for X rays (Tables 3-26 and 3-28).

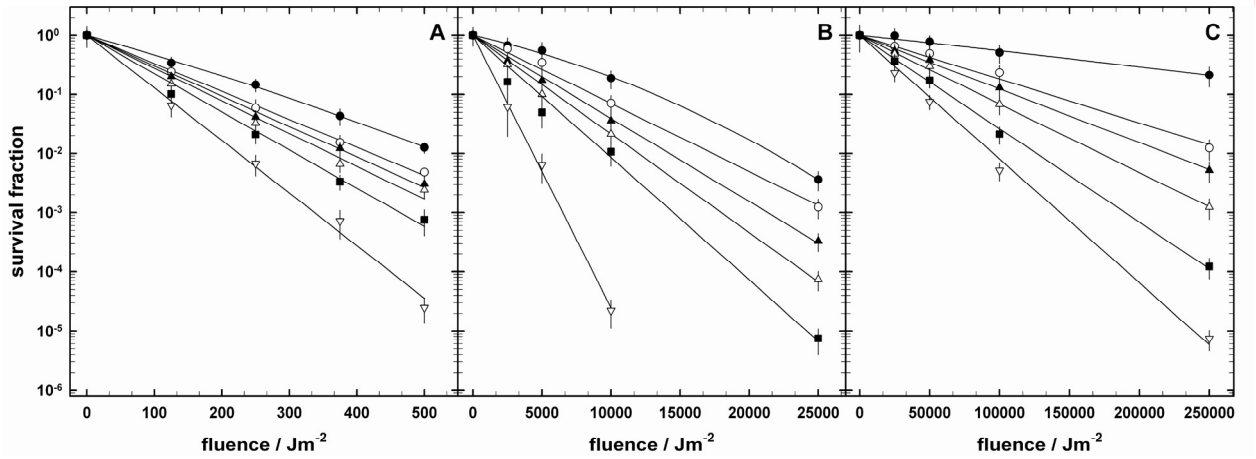


Fig. 3-33 Survival curves of *B. subtilis* spores in response to 254-nm UV-C (**A**), 290-400 nm UV-(A+B) (**B**) and 320-400 nm UV-A radiation (**C**). Strains are: 168 wild-type (solid circles), *recA* (open circles), *ykoU* (solid triangles up), *ykoV* (open triangles up), *ykoU ykoV* (solid squares) and *splB* (open triangles down). Data are expressed as averages and standard deviations (n = 3).

Relative to wild-type spores, in response to all UV wavelengths (UV-C, UV-A+UV-B, and UV-A), spores carrying *splB*-deficiency showed the highest rate of inactivation (ranging from 3- to 9-fold), followed by the *ykoU ykoV* double mutant, *ykoV* and *ykoU* single mutants, and the *recA*-deficient strain (Fig. 3-33A-C, Table 3-28). Interestingly, both DNA strand-break repairing mechanisms, recombination-mediated repair by *recA* and *ykoVU*-mediated NHEJ, became progressively more important to spore resistance in response to longer UV wavelengths (Table 3-28). These observations are in good agreement with the previous demonstration that exposure of spores to longer artificial or

solar UV-B and UV-A wavelengths produced a higher proportion of SSB and DSB in spore DNA (Slieman and Nicholson, 2000). The results suggest that NHEJ provides a major DNA strand-break repair strategy for spores irradiated with UV-B and UV-A wavelengths > 290 nm, such as encountered on Earth's surface (Nicholson et al., 2000).

Table 3-28 Survival curve characteristics of NHEJ-deficient spores after mono- and polychromatic UV

UV-C (254 nm)	F_{10} (J/m²)	F_0 (J/m²)	F_q (J/m²)	IC (m²/J)
B. subtilis (wild-type)	273.1 ± 42.3	122.1 ± 18.8	15.4 ± 6.7	$(9.65 \pm 0.67)\times10^{-3}$
recA	176.3 ± 29.1	86.2 ± 17.4	3.2 ± 1.0	$(1.37 \pm 0.22)\times10^{-2}$
ykoU	169.2 ± 18.0	74.7 ± 9.6	2.1 ± 0.5	$(1.48 \pm 0.28)\times10^{-2}$
ykoV	153.3 ± 24.6	62.3 ± 12.1	n.d.	$(1.57 \pm 0.19)\times10^{-2}$
ykoU ykoV	138.3 ± 15.2	50.2 ± 17.6	n.d.	$(1.78 \pm 0.31)\times10^{-2}$
splB	78.2 ± 13.1	25.4 ± 6.7	n.d.	$(2.35 \pm 0.12)\times10^{-2}$

UV-C (254 nm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.31 ± 0.07	1	0.998	
recA	0.91 ± 0.12	0.66 ± 0.11	0.993	0.046
ykoU	0.88 ± 0.08	0.61 ± 0.07	0.989	0.038
ykoV	0.76 ± 0.07	0.55 ± 0.06	0.990	0.035
ykoU ykoV	0.72 ± 0.05	0.49 ± 0.09	0.992	0.033
splB	0.69 ± 0.13	0.28 ± 0.07	0.984	0.014

UV-(A+B) (290-400 nm)	F_{10} (kJ/m²)	F_0 (kJ/m²)	F_q (kJ/m²)	IC (m²/J)
B. subtilis (wild-type)	11.3 ± 0.9	5.6 ± 0.7	1.3 ± 0.2	$(2.29 \pm 0.33)\times10^{-4}$
recA	8.9 ± 1.4	4.1 ± 1.0	0.5 ± 0.1	$(2.76 \pm 0.41)\times10^{-4}$
ykoU	6.8 ± 0.7	2.7 ± 0.4	n.d.	$(3.17 \pm 0.19)\times10^{-4}$
ykoV	5.6 ± 0.8	2.0 ± 0.4	n.d.	$(3.78 \pm 0.46)\times10^{-4}$
ykoU ykoV	4.2 ± 0.3	1.4 ± 0.3	n.d.	$(4.57 \pm 0.28)\times10^{-4}$
splB	2.0 ± 0.4	0.9 ± 0.2	n.d.	$(1.06 \pm 0.29)\times10^{-3}$

UV-(A+B) (290-400 nm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.47 ± 0.12	1	0.985	
recA	0.95 ± 0.11	0.79 ± 0.14	0.996	0.049
ykoU	0.89 ± 0.05	0.60 ± 0.06	0.994	0.042
ykoV	0.82 ± 0.09	0.50 ± 0.07	0.991	0.039
ykoU ykoV	0.70 ± 0.02	0.38 ± 0.10	0.993	0.025
splB	0.68 ± 0.05	0.19 ± 0.08	0.986	0.009

UV-A (320-400 nm)	F_{10} (kJ/m²)	F_0 (kJ/m²)	F_q (kJ/m²)	IC (m²/J)
B. subtilis (wild-type)	364.5 ± 39.2[a]	162.0 ± 21.3	8.4 ± 1.6	$(6.47 \pm 0.40)\times10^{-6}$
recA	136.4 ± 17.1	62.3 ± 7.8	5.9 ± 1.1	$(1.76 \pm 0.28)\times10^{-5}$
ykoU	110.2 ± 9.7	47.8 ± 5.9	0.8 ± 0.1	$(2.07 \pm 0.18)\times10^{-5}$
ykoV	86.8 ± 13.2	37.8 ± 7.2	0.4 ± 0.2	$(2.69 \pm 0.34)\times10^{-5}$
ykoU ykoV	62.1 ± 7.2	25.7 ± 3.6	0.2 ± 0.03	$(3.61 \pm 0.30)\times10^{-5}$
splB	43.9 ± 8.9	15.9 ± 2.4	0.1 ± 0.02	$(4.82 \pm 0.49)\times10^{-5}$

UV-A (320-400 nm)	n	RF	R^2	*P*
B. subtilis (wild-type)	1.23 ± 0.11	1	0.988	
recA	0.97 ± 0.08	0.44 ± 0.09	0.993	0.039
ykoU	0.91 ± 0.04	0.32 ± 0.08	0.998	0.033
ykoV	0.88 ± 0.07	0.26 ± 0.08	0.987	0.027
ykoU ykoV	0.75 ± 0.06	0.19 ± 0.06	0.996	0.024
splB	0.71 ± 0.04	0.14 ± 0.03	0.990	0.018

n.d. not determinable. [a]Value obtained by extrapolation. The ratio of the IC values of the repair deficient strains to those of the wild-type *B. subtilis* strain 168 is called "repair factor with regard to survival" (RF). *P* = statistical significance of difference of data of respective strain compared to *B. subtilis* wild-type strain 168; $P \leq 0.05$ = significant difference. Data are expressed as averages and standard deviations ($n = 4$).

Interestingly, the X-ray data closely mimicked the responses of the *recA*, *ykoU, ykoV,* and *ykoU ykoV* strains to UV-A (Fig. 3-31 and 3-33C) further supporting the notion that both UV-A radiation and X-rays produce substantial SSB and DSB in spore DNA. This contention is further strengthened by the

observation that spores of the *splB* mutant strain, which is specifically defective in repair of the UV-induced photoproduct SP, were not significantly more sensitive to X-rays than wild-type spores (Table 3-26). In conclusion, when faced with prolonged exposure to harsh environments, dormant bacterial spores must repair accumulated DSB during germination to ensure their survival and genome integrity. These results lend support to the hypothesis that NHEJ is a key strategy used during spore germination to repair DSB caused by terrestrial UV-B and UV-A radiation, as well as UV, ionizing radiation, and high vacuum-induced extreme desiccation encountered during exposure to space.

4. Discussion

4.1 Approaches to reach the aim of this work

It is the aim of this work to contribute to the understanding of mechanisms responsible for the high resistance of bacterial endospores to environmental stress parameters. Spores of *B. subtilis* 168 were used, because they are generally considered as ideal model for dormant microbial systems and because they are already extensively characterized, e.g., protein and genomic data bases are available. Standardized experimental protocols were used to allow intercomparison of different strains without any disturbing influences by the preparation and analyzing procedures, such as sporulation conditions or RNA isolation procedures. Standardization is especially critical for the sporulation process, because previous studies have demonstrated the dependence of spore resistance on the sporulation conditions, although the mechanisms are still not known (Horneck and Bücker, 1971; Setlow, 1995; Nicholson et al., 2000).

Physical stress parameters of environmental importance - either on Earth or in space - were selected as follows: UV radiation (UV-C at 254 nm and polychromatic UV-A), ionizing radiation (X-rays as well as accelerated heavy ions), long-term exposure to high vacuum, and high shock pressures simulating meteorite impacts. To obtain detailed information on the influences of those harsh terrestrial or simulated extraterrestrial conditions on the vitality of the treated spores, survival (based on CFA), induced mutations (genetic specificity or phenotypic morphology), DNA damage and transcriptional responses during spore germination were studied. To investigate the role of endogenous DNA protection in spore resistance, e.g. by SASPs (small acid-soluble spore proteins), the responses of wild-type spores were compared with those deficient in major SASPs. To study the repair capability of spores in coping with damage induced by the above mentioned treatments, the responses of wild-type spores and DNA repair-deficient spores were analyzed in parallel. Hence, after each treatment a combination of vitality measurements (spore survival), biochemical (DNA damage and mutagenesis), physiological (colony morphology) and genome-wide transcriptome analyses were applied to explore the physiological and transcriptional changes that occur during spore germination and outgrowth in response to the stress parameter exerted to the spores.

4.2 Impact of different stress conditions on the integrity of the spores

4.2.1 Inactivation

Damage to DNA, proteins or other essential components of bacterial spores poses a unique problem. Spores remain metabolically inactive during their state of dormancy (Nicholson et al., 2000), which may last for very long periods. In this dormancy state, substantial DNA and protein damage may accumulate and the spores are incapable of repairing and/or degrading such damaged DNA and proteins (Hayes et al., 1998). Repair of induced DNA (or protein) damage does not occur before the process of germination, when spores reactivate and prepare to return to vegetative growth (Setlow and Setlow, 1996).

All physical stress parameters tested led to inactivation of the wild-type spores. The survival data obtained after exposure of wild-type spores to UV or ionizing radiation are well in agreement with literature data (Baltschukat et al., 1986; Nicholson et al., 2000). Spores of various *Bacillus* species are generally 10- to 100-fold more resistant to UV than are the corresponding vegetative cells (Nicholson et al., 2000; Setlow, 2001). The major lethal target for UV radiation in spores is almost certainly DNA, as inferred from action spectroscopy (Munakata et al., 1991; Horneck et al., 1995; Setlow, 2001; Horneck and Rabbow, 2007). The efficiency of UV radiation in forming photoproducts in the DNA of the spores will be discussed in 4.2.2. Several cellular protective mechanisms have been identified to be involved in the spore resistance to UV, which will be further discussed in 4.3.

It is well established that also for ionizing radiation the spore DNA represents the major sensitive target (Baltschukat and Horneck, 1991; Micke et al. 1994). The sporicidal action increased in the order X-rays > Helium ions > Carbon ions > Silicium ions > Iron ions, confirming previous results that the spore inactivation is closely related to the LET of the applied heavy ion species. However, further irradiations with particles of energies > 200 keV/µm are needed to analyze the LET inactivation dependency.

Various authors mention the role of ring-like or toroidal-shaped genome structures in the radioresistance of microorganisms (Levin-Zaidman et al., 2003; Englander et al., 2004; Zimmerman

and Battista, 2005). As example they quote *Deinococcus radiodurans* with a more condensed genome than found in radiosensitive species. This pattern of condensation was not disturbed by ionizing irradiation (Zimmerman and Battista, 2005). It seems that highly ordered nucleoids allow higher DNA integrity and improved repair efficiency of DNA damage, which may support higher resistance to ionizing radiation. But, there is no direct correlation between the exhibition of toroidal DNA and being highly resistant to ionizing radiation as demonstrated by the radioresistant bacterium *Thermus aquaticus* (D_{20} = 1000 Gy for X-rays) with the genome uniformly distributed throughout the cell (Zimmerman and Battista, 2005). Toroidal DNA shape was also detected in dormant spores of *B. subtilis* and *B. megaterium* (Englander et al., 2004; Frenkiel-Krispin et al., 2004). This structure is closely connected with the presence of spore DNA binding proteins (SASPs) as will be discussed further in 4.3.

The nature of the sensitive target in spores is less clear for stress parameters that lead to extreme desiccation, like vacuum exposure, or to extreme shear forces and high temperature peaks, like the shock pressures associated with meteorite impacts. In spores *in vacuo* cellular water is gradually removed. As a result, hydrophilic bonds (in membranes and proteins) are disrupted (Potts, 1994). This leads to destabilization of the structures of proteins and DNA (Dose et al., 1992). Although these changes are lethal to most microorganisms, spores survive long periods of desiccation or vacuum, because they are able to stabilize their water-requiring structures by accumulating Ca^{2+}-DPA during sporulation, which is considered as a partial water replacement process (reviewed in Nicholson et al., 2000; 2005). Another major reason for spore resistance to vacuum-desiccation is protection of their DNA by SASP as will be discussed in 4.3. In the present work, roughly 20 % of the initial wild-type spore population was able to survive a 450 d lasting exposure to high vacuum (10^{-7} Pa; simulating space-like vacuum conditions of the low Earth orbit). The surviving vacuum-exposed spores did not show any difference in germination kinetics and RNA isolation efficiency compared to non-exposed controls, which hints to the fact that the cell envelops of the spores were not damaged by the vacuum treatment. A further protection of spores to vacuum was achieved by the addition of glucose, resulting in about 80 % survival after 6 years exposure to space vacuum (Horneck et al., 1994a). Non-reducing sugars, such as trehalose or sucrose are commonly used desiccation protectants in anhydrobiotes, (reviewed in Potts, 1994; Manzanera et al., 2004). These molecules help to prevent damage to the

DNA, membranes, or proteins by replacing the water molecules during the desiccation process and thereby preserving the three-dimensional structure of the biomolecules (Crowe and Crowe, 1992). However, this strategy is not known for spores. In spores, neither the mechanism for DNA protection nor the nature and distribution of the DNA and protein damage caused by extreme desiccation or vacuum exposure are known.

Shock recovery experiments were performed with spores of *B. subtilis* to simulate the meteorite impact and ejection process as first step of the scenario of "*Lithopanspermia*" (Mileikowsky et al., 2000; Nicholson et al., 2000; Horneck et al., 2001b). To mimic an artificial endolithic bacterial sample – an arrangement required for "*Lithopanspermia*" - spores in dry layers were mounted between two gabbro plates when exposed to shock pressures in the range of 5 GPa to 50 GPa, which corresponds to shock pressures determined for Martian meteorites (Fritz et al., 2005; Horneck et al., 2007; Stöffler et al., 2007). The survival rate decreased exponentially with pressure up to 42 GPa (10^{-4} survival rate). At a shock pressure of 50 GPa the spore survival dropped below the detection threshold (depending on the initial spore population of 10^8 spores/sample). In these shock recovery experiments two physical parameters – shock pressure and/or steep temperature increase (Stöffler and Langenhorst, 1994) - may be responsible for spore inactivation. Rock temperature increases up to several 100°C have been calculated, although lasting for fractions of μs only (Horneck et al., 2007). Whereas the spore envelops might be the sensitive target for high shock pressures, high temperature may affect the integrity of the DNA. To differentiate between the effects of both parameters associated with shock recovery experiments, the experiments were repeated with the same spore arrangement, however at a lower pre-shock temperature (193 K instead of 293 K). At that low pre-shock temperature, a certain fraction of spores (10^{-4}) survived shock pressure at 50 GPa, which had led to complete inactivation at room pre-shock temperature (293 K). These results indicate that, in addition to the pressure, peak or post shock temperatures may be decisive in spore inactivation during a meteorite impact event. Calculations have further shown that temperature increases are directly related to the water content of the biological layer (Horneck et al., 2007). Using air-dried or moisturized spore layers in an identical experimental setup, it was found that at a shock pressure of 30 GPa moisturized spores - which had experienced higher temperature increases - were inactivated about 10 times more efficiently than air-dried spores. This

result further confirms the lethal role of high temperatures associated with meteorite impacts and that DNA may be one of the critical targets of spores in the first step of "*Lithopanspermia*".

4.2.2 DNA damage

DNA is considered to be the most important radiosensitive target in biological systems. Whereas UV-C predominantly induces characteristic nucleotide modifications, mainly pyrimidine photoproducts, via direct absorption of photons in the DNA bases (Cadet et al., 2005), UV-A as well as ionizing radiation cause damage to the DNA either directly or indirectly via radiation-induced free radicals, mainly radiation-induced radical oxygen species (ROS) (Hutchinson, 1985; Dianov et al., 2000). The data of the thesis confirm previous reports that the predominant DNA photoproduct in UV-C irradiated *B. subtilis* wild-type spores is SP (Varghese, 1970), which increased linearly with the UV-dose. Furthermore, with the advanced technique of HPLC-MS/MS a systematic quantitative fluence-effect relation was established separately for the different bipyrimidine photoproducts of UV-C or UV-A irradiated spores, which all showed linear induction curves.

It is interesting to note that SP was also the main pyrimidine photoproduct caused by the less energetic UV-A radiation, whereas most studies so far have concentrated on 8-oxodGuo caused by an indirect UV-A effect (Ravanat et al., 2001). This finding might have environmental health implications in view of the fact the UV-A contributes the major portion to the terrestrial UV-spectrum. Slieman and Nicholson (2000) reported on DNA strand-break (SSB and DSB) induction in *B. subtilis* spore DNA by solar UV, probably generated by interactions of ROS with spore DNA. It might be interesting to compare the efficiency in the induction of different types of photoproducts by UV-A (direct *versus* indirect effect) which was however beyond the scope of this work.

DNA strand breaks are the major lesions in the genome of spores exposed to ionizing radiation (Micke et al., 1994), caused either directly by the highly energetic photons or accelerated particles or indirectly via interaction of radiation-induced free radicals, e.g. ROS, with the DNA (Hutchinson, 1985; Cadet et al., 1999; Dianov et al., 2000). Several radiobiological studies at accelerators and in space experiments lead to the assumption that at least two qualitatively different lesions are formed depending on the LET of the heavy ion irradiation. Damage produced by low LET (< 2 keV/µm) particles can be attributed to

ionization and excitation processes or interactions with radicals. DNA strand breaks, base damage/modification or crosslinking between bases or DNA and protein are likely to occur. For high LET (> 2 keV/µm) particles additional physicochemical processes, such as thermal spikes or shock waves, have been conjectured and may cause local destruction of ordered structures (Horneck and Bücker, 1983) and direct ionization of DNA bases and/or indirect interaction of ionizing radiation-induced ROS with DNA bases, which led to e.g. to 8-oxodGuo formation (Douki et al., 2006). Concerning low LET radiation like γ-rays, it typically causes about 80 % of DNA damage indirectly via ROS, whereas the remaining 20 % of damage occur by direct interactions of the photons with the DNA (Halliwell and Gutteridge, 1999). This ratio changes with increasing LET of the radiation, such as accelerated heavy ions, towards a higher fraction of direct effects (Baltschukat and Horneck, 1991; Kramer and Kraft, 1994; Cadet et al., 1995; Shikazono et al., 2002). Whereas the induction of DNA strand breaks has been extensively studied for radiations of different qualities, little is known on the production of oxidative damage in the DNA bases. Those base changes were subject of this study. 8-oxodGuo as well as ThdGly increased linearly with dose. The efficiency of their induction increased with LET of the radiation. Again, a comparison of the induction of different DNA damage by different radiation qualities was beyond the scope of this study that was mainly tackling the question how spores during germination cope with DNA damage caused by different environmental stressors.

According to Dose et al. (1991; 1992) the DNA of vacuum-exposed spores of *B. subtilis* is damaged to a very substantial degree by processes leading to DNA-strand breaks (about 50 strand breaks per genome after three weeks vacuum at about 10^{-4} Pa). So far, little is known about other types of DNA damage caused by vacuum exposure in spores. Likewise, no direct evidence has been obtained on the type of DNA damage - if any - caused in spores subjected to shock recovery experiments simulating meteorite impacts. Due to the fact, that there are no details on the nature of shock pressure-induced damage(s) in the spores, only some presumptions can be made: In the experiments spores are encased between two slides of rock material, which may lead to mechanical stress (mainly abrasions) during the explosion. Furthermore, the rock-microorganisms-sandwich was embedded in a steel-container, which is a optimal heat-conductor, the high temperatures which occurred within the progression of the (terminal) shock pressure may have led to (dry) heat-generated DNA strand breaks. However, further

studies on the nature of high pressure-induced damage(s) and putative DNA repair/protection(s) strategies are needed.

4.2.3 Mutational specificity

Despite their remarkably high resistance to desiccation or ionizing radiation, *B. subtilis* spores manifested different types of DNA damage when being kept in an extremely dry environment (produced by high vacuum) or exposed to ionizing radiation. If the integrity of the DNA is not completely restored, DNA damage may lead either to lethality or to mutations. Mutagenesis to azide resistance or histidine reversion in heavy ion irradiated spores of *B. subtilis* has been reported (Baltschulat and Horneck, 1991; Kiefer, 2002; Horneck et al., 1994b) as well as to nalidixic acid resistance in vacuum-exposed spores (Munakata et al., 1997), indicating that DNA is targeted by both treatments.

In this work, the induction of mutations to rifampicin resistance (Rif^r) in spores of *B. subtilis* exposed either to high vacuum or to ionizing radiation was investigated. The spontaneous mutation rate of about 5×10^{-9} determined for spores of *B. subtilis* 168 is well in agreement with literature data (Nicholson and Maughan, 2002) and is about half the value given for vegetative cells. It was found that Rif^r-mutations increased exponentially with the dose of ionizing radiation and the mutation induction rate increased with increasing LET. This LET dependence was also observed for the induction of oxidative damage in the DNA of irradiated spores, however in the latter case the induction increased linearly with dose. Therefore, is not possible from the current data to establish a relation between Rif^r mutations and DNA damage caused by irradiation of spores. Rif^r mutations were also induced in vacuum-exposed spores at a rate increasing with exposure time.

Because the site of the Rif^r mutation is well characterized - it is located in the *rpoB* gene encoding the β subunit of a RNA polymerase, with the majority (> 90 %) of those mutations occurring in a short stretch which corresponds to the Rif^r cluster I in the *E. coli rpoB* gene (Nicholson and Maughan, 2002) – the mutational specificity of 30 Rif^r mutations induced in *B. subtilis* 168 spores either by ionizing radiation or high vacuum was analyzed. All induced mutations were caused by base changes, either transitions (43.3 %) or transversions (46.7 %) and only 7 % of the mutations were located outside of cluster I, however within *rpoB*. Four mutational hot spots were observed in cluster I: A-to-T

transversion resulting in Q469L; A-to-C transversion resulting in H482P; C-to-T transition resulting in A478V; and C-to-T transition resulting in S487L. The most frequent changes, Q469L and H482P, are already known as the major type of spontaneous Rif^r-mutations (Nicholson and Maughan, 2002) as well as induced in vacuum-exposed spores (Munakata et al., 2002; 2004). Additionally to the already previously characterized *rpoB* changes, four Rif^r-mutations in cluster I arising from irradiated spores were first described in this work: L467P, A478V, R484P and A488P, as well as two codon changes R504H and H507R located between cluster I and II (Campbell et al., 2001). Further genetic transformation experiments of the described *rpoB*-Rif^r sequences in rifampicin-sensitive strains of *B. subtilis* 168 will be needed for verifying that these newly described *rpoB* mutations are indeed causing a Rif^r phenotype.

This proof of mutagenic modifications induced in the genome of bacterial spores by ionizing radiation or high vacuum may stimulate further studies on the role of the mutagenic force in the evolution of microorganisms living and growing under continuous exposure to high doses of ionizing radiation. Habitats with a high background radiation may lead to different mutational spectra for antibiotic resistance in radiosensitive or radioresistant microorganisms.

Likewise the role of extreme dryness in microbial evolution needs to be assessed. In summary, this work has shown that in a dormant bacterial endospore its DNA is the major target of ionizing radiation as well as of high vacuum exposure, and induced base modification may lead to changes in the physiology of the progeny; one well studied aspect hereby is increased resistance to known chemicals such as antibiotics.

In addition to direct effect of extreme shock pressure, i.e. inactivation, changes in the colony morphology probably caused by sporulation deficiency were determined, which leads to the assumption that exposures to high pressure have a mutagenic influence on the spore genome (3.6.2). Interestingly, spores which were tested for their survival after a hypervelocity atmospheric entry (stage 3 of the "*Lithopanspermia*" hypothesis, the nondestructive deposition of biological material on another planet) exhibited a similar increase in the induction of sporulation-defective cells (Fajardo-Cavazos et al., 2005).

4.3 Role of SASPs in DNA protection of spores

An essential step during sporulation is the association of the DNA with a family of small, acid-soluble proteins (SASPs) which leads to the transformation of the DNA into a toroid-like structure. SASPs bind largely to the outside of the DNA helix and straighten and stiffen it thereby changing its structure from the B-form to an A-like helix, in which it is largely protected against many types of DNA damage (Setlow, 1995). 16 small, acid-soluble spore protein encoding genes (*sspA-sspP*) were found in the *B. subtilis* 168 genome (Kunst et al., 1997; Moszer et al., 2002).

When comparing the survival of wild-type spores with those of mutants deficient in α/β-type SASPs (*sspA sspB*), it was convincingly shown that the mutants exerted a significantly higher sensitivity to all radiation qualities tested: UV-C, UV-A, as well as ionizing radiation. Spores lacking the major SASPs exhibited a 5- to 10-fold higher inactivation by UV-C or UV-A radiation than wild-type spores, and they were about 3 times more sensitive to X-rays or accelerated heavy ions at an LET range of 2 - 200 keV/µm than their wild-type counterparts. This increased sensitivity of *sspA sspB* spores to different qualities of radiation provides further support to the supposition on the important role of SASPs in spore resistance by stabilizing the DNA (Table 4-1).

Table 4-1 Role of major SASPs, SP lyase and NHEJ in the spore resistance to different treatments as determined in this work (or taken from literature) by use of mutants deficient in those features.

Treatment	α/β-type SASPs (*sspA sspB*)	SP lyase (*splB*)	NHEJ (*ykoU ykoV*)
UV-C	+	+	–/+
UV-A	+	+	+
Ionizing radiation	+	–	+
High vacuum/desiccation	–/+ (Setlow, 2006)	–	+

Symbols: + high importance; – no effect; –/+ some importance but has some effect; (?) no data available.

An essential consequence of the SASPs–DNA binding complex of spores is the spectrum of UV-induced photoproducts, which shows a shift from that observed in vegetative cells (i.e. mainly CPDs and 6-4PPs) towards a nearly exclusive induction of SPs (Varghese, 1970; Moeller et al., 2007a). The detailed analysis of all bipyrimidine photoproducts by HPLC-MS/MS confirmed that only little if any

CPDs or 6-4PPs were formed in wild-type spores after irradiation with UV-C or UV-A. In contrast, *sspA sspB* spores gave less SPs than wild-type spores upon UV-C or UV-A irradiation, but a significant level of CPDs and 6-4PPs, as has already been shown by Douki et al. (2005a; 2005b) and Moeller et al. (2007a). It is important to note that the yield of all bipyrimidine photoproducts per chromosome was not significantly different for both strains, only their spectrum had changed. These observations further confirm that the most important factor in the spore UV resistance is the saturation of spore DNA with SASPs.

In contrast to the situation with UV radiation, where a substantial amount of data is available on the role of spore features, such as spore coat layers, Ca^{2+}-DPA-chelate complex and SASPs, in spore UV resistance (Nicholson et al., 2000; 2005; Setlow, 2006), less is known on the impact of those spore features on ionizing radiation effects in spores. In this work it was found that in *sspA sspB* spores about twice as much oxidative DNA damage, measured as 8-oxodGuo and ThdGly, was induced by particle radiation than in equally treated wild-type spores. These data, together with those of a 3-fold higher inactivation of *sspA sspB* spores by ionizing radiation lead to assumption that SASPs play a major role in spore resistance to ionizing radiation by either preventing direct damage to the DNA through stabilization of its integrity or by scavenging radiation-induced ROS. Systematic further studies on the role of spore components e.g. sulfur-rich spore coat proteins and/or SASPs are required to provide more information on their role in spore resistance to ionizing radiation, especially in the prevention of radiolysis-induced spore DNA damage.

In addition to the DNA saturation with α/β-type SASPs, the structure of DNA-SspC complex is mainly involved in full coating of the spore DNA up to the point of tight packing of nucleoprotein helices, which directly enhance and complement the DNA protection by protruding dense assembly of adjacent DNA-SspC filaments (Frenkiel-Krispin et al., 2004). The role of this DNA-SspC complex in spore resistance to different types of radiation or other stress factors remains to be determined.

Recapitulating, it can be stated that α/β-type SASPs are involved in the general spore survivability to different radiation and pressure treatments, as determined in this work and summarized in Table 4-1.

4.4 Role of repair systems in coping with DNA damage induced in dormant spores

As outlined earlier, spores possess two possible methods for minimizing deleterious effects of DNA damaging treatments: (i) by protecting dormant spore DNA from damage in the first place, as was discussed in 4.3, and (ii) by ensuring rapid repair of DNA damage when spores germinate (i.e. return to vegetative life). There is probably a synergetic effect of both strategies for ensuring the unique high spore resistance and survival as has been shown by Setlow (1992; 1995). Despite the protection mechanisms, potentially lethal or mutagenic damage may eventually accumulate in the DNA of spores during their exposure to environmental stresses. Therefore, one further major determinant of the degree of spore resistance to extreme environments is the speed and accuracy with which spore DNA damage is repaired during germination. It is one of the main goals of this work, to contribute to the understanding of the role of specific DNA repair pathways in coping with DNA damage caused by environmental stress factors in spores. There is especially relatively little information on the repair of DNA damage induced by ionizing radiation in spores.

4.4.1 Responses of repair-deficient mutants to cope with DNA damage induced in spores

Several DNA protection and repair mechanisms e.g. SASPs, SP lyase and NHEJ (under σ^G-regulation) are already synthesized during sporulation to ensure DNA stability as well as quick DNA repair of spore-specific damage after germination activation, whereas other repair systems, such as nucleotide excision repair (NER) and recombination-mediated repair (RR) need resynthesis during germination. An appropriate method to study the efficiency of different repair systems is to compare the responses of repair deficient spores with those of their wild-type counterparts. The degree in sensitivity of repair deficient spores is determined by the type of damage caused by the treatment and the specificity of the repair system under investigation. In this work, the following repair pathways were studied: recombination-mediated repair by using a *recA* mutant, the SP specific repair by using the *splB* mutant, the SP specific repair in combination with NER by using the *splB uvrB* double mutant, and all three pathways by using a *recA splB uvrB* mutant. All mutants were descendents of the same strain, i.e. isogenic in all other markers.

Deficiencies in those repair genes led to a significant decrease in spore resistance to UV, ionizing radiation or extreme desiccation. Concerning UV-C radiation, the resistance of spores, taken from the

RF values (repair factor with regard to survival), decreased in the following order: wild-type > *recA* > *splB* or *splB uvrB or recA splB uvrB*. In the *recA* mutant, the RF value amounted to about 50 % of that of the wild-type spores. Statistically identical RF values were obtained for all three SP lyase deficient mutants, which reached about 30 % repair efficiency compared to the wild-type spores. This trend is also reflected in the bipyrimidine PP repair efficiency of the different strains. Whereas the total yield of UV-C induced PP was statistically identical for all tested strains, after 60 min germination in wild-type spores about 70 % of the PP had disappeared, whereas the numbers were about 50 % for the *recA* mutant, and 15 - 19 % for the SP lyase deficient mutants. This trend is even more aggravated when comparing the repair efficiency of SP, which in the SP lyase deficient mutants amounted to 6-8 % of that in wild-type spores. These data confirm the overwhelming role SP lyase plays in spore resistance to UV-C radiation (Table 4-1). It can hardly be replaced by any of the other repair pathways tested. A similar trend was observed for UV-A radiation. With RF values between 14 % and 27 % the three SP lyase deficient mutants showed again the lowest repair efficiency.

With regard to ionizing radiation, the *recA* mutants reached about 20 % repair efficiency compared to wild-type spores, irrespective of the radiation quality the spores were exposed to. This is much lower than after UV-C radiation (50 %) or UV-A radiation (70 %) and emphasizes the important role of recombination-mediated repair for DNA damage induced by ionizing radiation. The RF value of the SP lyase deficient mutant *splB* was quite high (75 ± 17 %) compared to the wild-type spores. This was expected, because the target of SP lyase is the UV specific SP damage in DNA, which is hardly – if at all – induced by ionizing radiation. Hence, its role in repair of DNA damage induced by ionizing radiation in spores is negligible (Table 4-1).

Previous studies of spores exposed to extreme desiccation via high vacuum indicated that wild-type spores were quite resistant while DNA repair-deficient spores showed significant sensitivity to extended dryness via vacuum (Munakata et al., 1997). This observation was confirmed in this work with RF values of 20 % for the *recA* mutant and about 50 % for the *splB* mutant. This high sensitivity of the *recA* mutant to extended vacuum exposure is another indication that the DNA is the sensitive target of vacuum treatment. It might even be suggested that vacuum-induced DNA damage may be of

similar nature than that induced by ionizing radiation. DNA strand breaks may be the major lethal damage caused by vacuum.

In Table 4-2 different types of radiation- and pressure-induced DNA damage in dormant spores of *B. subtilis* and the respective DNA repair pathway(s) and protective role of major SASPs are shown and summarized.

Table 4-2 Efficiency of DNA repair pathway and protection mechanism to prevent or repair different types of induced spore DNA damage in dormant spores as determined in this work by use of mutants deficient in those features.

DNA repair pathway / DNA protection mechanism	RR (*recA*)	NHEJ (*ykoU ykoV*)	SP lyase (*splB*)	NER (*uvrB*)	SASPs (*sspA sspB*)
DNA PP (6-4PPs, CPDs)	–/+	(?)	–/+	+++	–/+
DNA PP (SP)	–/+	0	+++	+	+++
DNA strand breaks (SSB, DSB)	+	+++	0	(?)	–/+
oxidative DNA damage	–/+	–/+	(?)	(?)	+

Symbols: +++ high importance; + minor importance; 0 no effect; –/+ some importance but has some effect; (?) no data available. 6-4PPs (6-4) photoproducts; CPDs cyclobutane pyrimidine dimers; SP spore photoproduct; SSB single single-strand breaks; DSB double-strand breaks.

4.4.2 Transcriptional responses during germination of stress-exposed spores

To obtain detailed insights in the transcriptional regulation in response to DNA damage induced in spores by different types of radiation or vacuum, the transcriptional level of treated and non-treated (control) spores during germination was determined via global gene expression analysis by cDNA microarray technique. Global gene expression profiles of damaged and non-damaged *B. subtilis* spores were obtained after 60 min germination, a time point during spore germination and outgrowth when the first round of chromosome replication is initiated and/or begins to start in intact spores (Siccardi et al., 1977; Eident-Wilkinson et al., 1992; Harry, 2001). At this critical stage in spore germination and outgrowth, the majority of the induced (DNA) damage has to be removed or at least initiated to be repaired to ensure error-free genome replication (Setlow and Setlow, 1996; Setlow, 2006; Keijser et al., 2007).

In response to UV-C or UV-A radiation, when significant amounts of DNA bipyrimidine photoproducts are generated by the photons absorbed in the DNA, specific (DNA and non-DNA) damage-responsive transcripts were significantly up-regulated. These are genes encoding the nucleotide excision repair mechanisms (*uvrA* and *uvrB*), putative UV endonuclease (*ywjD*), a UV-responsive UvrD-like ATP-depending DNA helicase (*yjcD*). Earl et al. (2002) described the UV damage response of the UV endonuclease, called *uvsE*, in *Deinococcus radiodurans*, which is highly similar to sequence of *ywjD* identified in this work. Veaute et al. (2005) reported that *uvrD/rep* DNA helicase acts as RecA nucleoprotein filament remover which is indirectly involved in DNA strand break repair mechanism (Carrasco et al., 2004).

After exposure to ionizing radiation, where single-strand breaks, double-strand breaks (both caused by direct energy absorption), base modification, abasic sites and sugar modifications (indirect radiation effect caused by interactions with ROS radicals) (Hutchinson, 1985; Douki et al., 2006; Qui et al., 2006) are induced, genes responding to DNA strand breaks (single-strand DNA-binding proteins: *hbs*, *ssb* and *ywqH*; DNA ligases: *ligA* and *ligB*) were up-regulated as well as genes that are directly involved in DNA repair of oxidative damage (*ytkD*, a mutT/nudix-like hydrolase), implicating the essential role in the DNA mismatch repair of oxidized bases (e.g. 8-oxo-7,8-dihydro-2'-deoxyguanosine) as has been pointed out by Bessman et al. (1996) and Xu et al. (2004). DNA depurination may indirectly result in DNA single-strand breaks. Interestingly the recently described apurinic and apyrimidinic endonuclease IV (AP) encoding gene *yqfS* (Kanno et al., 1999; Salas-Pacheco et al., 2005) was found to be induced in germinating spores after exposure to ionizing radiation. Data gathered in this work confirm earlier observations (Goranov et al., 2006; Whitehead et al., 2006) that equal types of DNA damage, e.g. DNA strand breaks caused by different treatments, such as UV-A radiation, ionizing radiation or extreme desiccation (Hutchinson, 1985; Dose et al., 1991; 1992; Slieman and Nicholson, 2000) have similar effects on the global transcriptional profile, as can seen by the induction of single-strand DNA-binding proteins to ensure structural DNA integrity.

Summing up, the majority of the up-regulated DNA repair and recombination genes identified in this work ensure genomic integrity and successful DNA repair of damage, primary of DNA strand-breaks. They include a large group of up-regulated "*y*"-genes encoding site-specific recombinases / resolvases

(Table 4-3), which are known to be involved in the repair of DNA damage (Zahradka et al., 2002; Au et al., 2005; Goranov et al., 2006) during DNA replication.

Table 4-3 Stress induced resolvase/recombinase-like encoding genes after 60 min spore germination

Gene	Function / Operon	Homolog to	UV-C	UV-A	Ionizing radiation[1/2]	High vacuum
ydcL	site-specific recombinase, phage integrase family / *ydcNML*	*Enterococcus faecalis* V583			+[1/2]	
yefB	site-specific recombinase (resolvase family) / *yefB*	*Geobacillus kaustophilus* HTA426		+	+[1/2]	+
yefC	site-specific recombinase, (resolvase family) / *yefCyeeABC*	*B. cereus* G9241		+	+[1/2]	+
*yjcR**	resolvase-like helix-turn-helix domain protein / *yjcRSyjdA*	*Solibacter usitatus* Ellin6076	+			
yneB	resolvase, N-terminal domain - putative recombinase / *yneABynzCtkt*	*B. licheniformis* ATCC 14580	+	+	+[1/2]	+
yopP	site-specific recombinase, phage integrase family / *yoqMNOPQR*	*Staphylococcus epidermidis* RP62A			+[1]	
<u>*yomM*</u>	site-specific recombinase, phage integrase family / *yonH-yonAyomZ-yomM*	*Staphylococcus epidermidis* RP62A	+			
yokA	resolvase, N-terminal: recombinase / *yokAypqP*	*B. weihenstephanensis* KBAB4		+	+[1/2]	+
yrrK	Holliday junction resolvase-like protein / *alaSyrzLyrrKyrzB*	*B. subtilis* subsp. *subtilis* str. 168	+	+	+[1/2]	+

Genes are listed by map position. The underlined genes are part of the prophage SPβ chromosome in *B. subtilis* 168 genome (Kunst et al., 1997). *Resolvase/recombinase with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). [1/2] Ionizing radiation exposure: [1] X-rays and accelerated Fe-ions ([2] Fe 500 MeV/n).

A biochemical model for homologous recombination in *B. subtilis* 168 is shown in Fig. 4-1 (as summarized in Sonenshein et al., 2002). The final step in homologous recombination is the resolution of the Holliday junction (Holliday, 1964). In *E. coli* the Holliday junction resolvase activity is catalyzed by the RuvC protein (West, 1996), which cleaves the pair of strands that form the non-crossover strands resulting in nicked duplex products (Connolly et al., 1991). Prior to resolution of the Holliday junction, the *E. coli* RuvA and RuvB proteins act together, presumably with RecA, to promote ATP-dependent branch migration (Tsaneva et al., 1992). However, almost nothing is known about

resolvase-/recombinase-like acting homolog(s) in *B. subtilis* and its/their function(s) in the (SOS-induced) recombination-mediated DNA repair of radiation- and extreme desiccation-caused damage (according to Au et al., 2005; Symington, 2005; Goranov et al., 2006). However, this homologous recombination pathway, which requires at least two homologous chromosomes, cannot operate on DSB during spore germination (Wang et al., 2006), because spores of *B. subtilis* contain a single chromosome only, arranged in a toroidal shape (Ragkousi et al., 2000; Frenkiel-Krispin et al., 2004). Therefore other mechanisms of DNA strand break repair need to be considered, such as NHEJ, which will be discussed in 4.4.4.

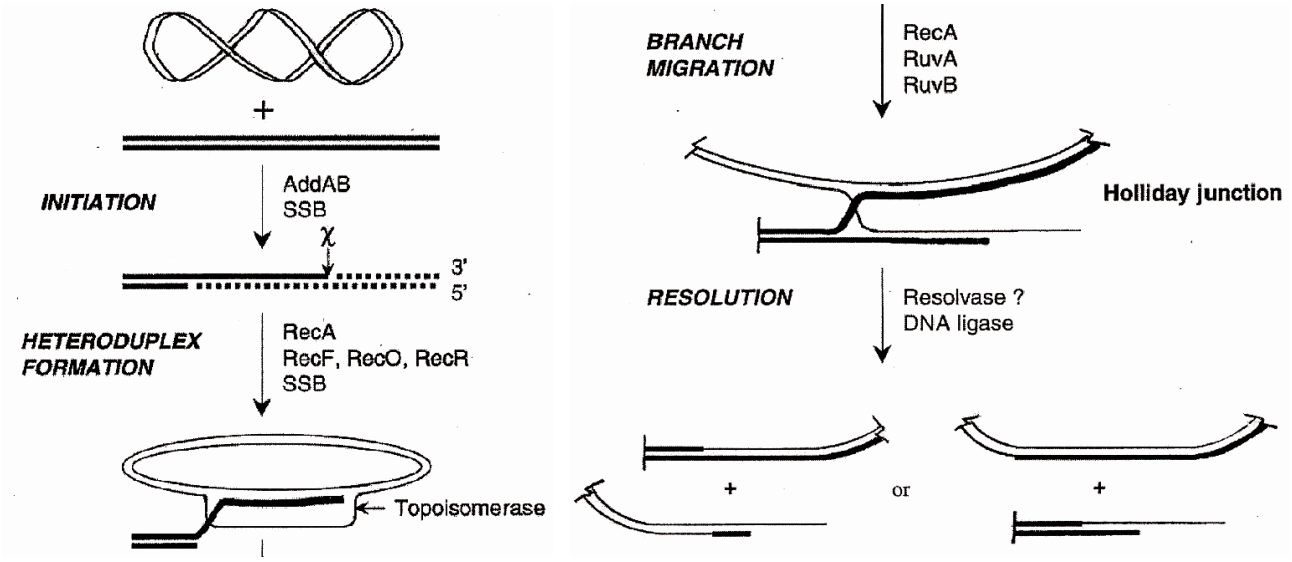


Fig. 4-1 Biochemical model for homologous recombination in *B. subtilis* 168 (adapted from Kowalczykowski et al., 1994)

Exposure of prokaryotes to DNA-damaging agents e.g. UV radiation, results in the induction of a diverse set of physiological responses, collectively called the SOS response (reviewed in Little and Mount, 1982; Friedberg et al., 1995). These responses include enhanced capacity for excision repair (e.g. BER and NER after base modification through e.g. oxidation and alkylation), enhanced mutagenesis and recombination-mediated repair and inhibition of cell division (by e.g. filamentation and/or phage induction) (Dubnau and Lovett, Jr., 2002). Induction of the SOS responses is due to the coordinated induction of the SOS genes and/or *din* (damage-inducible) genes (Yasbin et al., 1992). Several *din*-genes (according to Dubnau and Lovett, Jr., 2002) were found in this work to be up-

regulated during germination after DNA damage-induction in the spores. These are as follows: *recA*, *lexA*, *uvrB*, *ruvA*, *ruvB*, *dinB*, *tagC* (former *dinC*), *yolD* (putative chromosome segregation protein or cyclic nucleotide-binding domain containing protein) and *yqjW* (a homolog of the *E. coli umuC*, required for translesion, error-prone, DNA synthesis/repair) (Friedberg et al., 1995). By considering that recombination-mediated repair is an important mechanism for repairing two-strand DNA damage and single-strand non-base pairing lesions that encounter a moving replication fork (Friedberg et al., 1995), it is not unusual that several *din*-genes code for recombination-mediated repair leading to the assumption that several DNA repair genes have (at least) bifunctional activities in the fundamental cellular process, DNA repair. The *din*-genes are controlled by two proteins (which are themselves products of *din*-genes): the LexA protein, which represses the transcription of *din*-genes, and the RecA protein, which is activated by a metabolic signal to stimulate the proteolytic autodigestion of LexA (Little et al., 1981; Friedberg et al., 1995). The identification of RecA and LexA proteins in a wide range of bacteria indicates that SOS regulation has been conserved in prokaryotes (Dubnau and Lovett, Jr., 2002).

Recombination-mediated repair (recombinational repair) is known as the repair mechanisms of DNA lesions through a process similar to homologous recombination by using the same (recombination) enzymes e.g. recombinases (Sassanfar and Roberts, 1990; Kuzminov, 1995; Smith, 2004). In Table 4-3, a summary of the up-regulated resolvase/recombinase-like encoding genes after 60 min spore germination, their sequence homology and type of the inducible treatment are shown. Zahradka et al. (2002) showed that the RuvC resolvase in *E. coli* acts in the early stage of recombinational repair by processing and stabilizing the Holliday junction to ensure DNA replication by promoting the restoration of collapsed replication forks. However, the involvement of resolvases/recombinases in the recombination-mediated repair of *B. subtilis* is poorly understood. To obtain more information further studies on the genetic basis and functionality of these recombination enzymes in the DNA repair during germination of treated spores are needed.

Already in 1971, Gass et al. (1971) found that *polA*-deletions in *B. subtilis* lead to significant increase in the general sensitivity to DNA damaging agents e.g. UV. In 2003, Sung et al. (2003) reported about the role of homologs of *E. coli* UmuC/DinB or Y superfamily of DNA polymerases in their

involvement in SOS-induced mutagenesis. The DNA damage-inducible UmuD and UmuC proteins are required for another type of SOS mutagenesis in *E. coli* (Sommer et al., 1993). UmuCD-dependent translesion DNA synthesis allows cells to replicate past DNA damage-induced lesions that would normally block the continuing polymerization by the major replication DNA polymerase (DNA polymerase III). This translesion synthesis results in an increased mutation rate (Kato and Shinoura, 1977; Steinborn, 1978; 1979). It has become apparent that UmuC is the founding member of a superfamily of novel DNA polymerases that can replicate over lesions and/or operate in particular classes of imperfect DNA templates (Sutton et al., 2000). Both DinB (a template-directed, DNA-dependent DNA polymerase) and UmuC belong to this superfamily, which has been designated the Y family of DNA polymerases (Ohmori et al., 2001). To date, the UmuC protein of *E. coli* is unique within the Y family in that its polymerase activity is managed and/or activated by the products of the *umuD* gene (Sutton et al., 1999). UmuC, when complexed with UmuD, can replicate over abasic sites (Tang et al., 2000), thymine-thymine cyclobutane dimers, and 6-4 photoproducts (Tang et al., 1999). DinB protein can replicate templates possessing AP (apurinic-apyrimidinic) lesions in the template strand, often causing - 1 frameshift mutations (Wagner et al., 1999). The function and the different types of DNA polymerases involved in the DNA replication of *B. subtilis* are shown in Fig. 4-2.

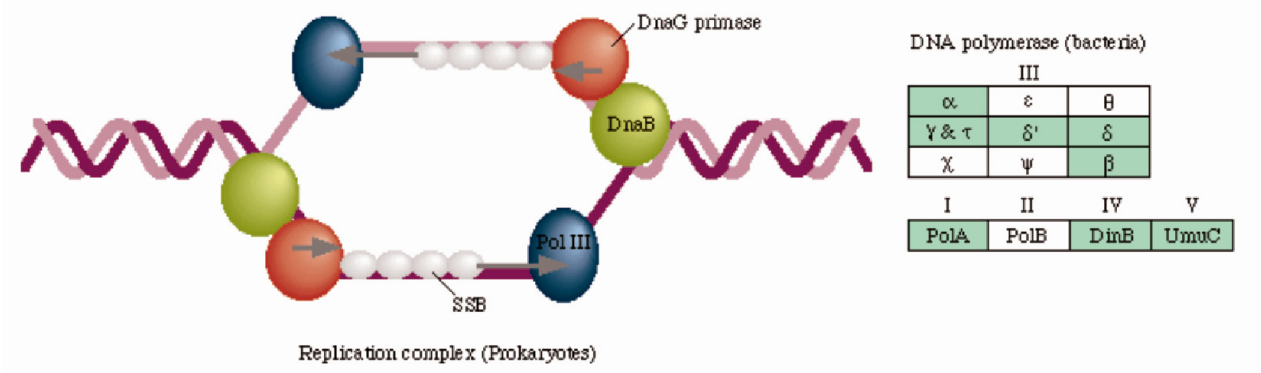


Fig. 4-2 Different kinds and classes of DNA polymerase of *B. subtilis* 168 (according to the KEGG PATHWAY Database; Kanehisa and Goto, 2000)

In this work, several DNA polymerases were significantly up-regulated after DNA damage-inducing treatment followed by 60 min spore germination (Table 4-4).

Table 4-4 Radiation- or high vacuum-induced DNA polymerases (including type and/or subunit classification) during spore germination of spores of *B. subtilis* 168

Ionizing radiation[1/2]	UV-A	UV-C	High vacuum[3/4]
polC (pol. III, alpha)[1/2]	*dnaN* (pol. III, beta)	*dnaX* (pol. III, gamma, tau)	*dnaX* (pol. III, gamma, tau)[3/4]
yobH (pol. IV, DinB)[1/2]	*polC* (pol. III, alpha)	*yobH* (pol. IV, DinB	*ykuL* (pol. I, PolA)*[3/4]
yosH (pol. III, alpha)*[1/2]	*yobH* (pol. IV, DinB)	*yozK* (pol. IV, DinB	*ymzC* (pol. III, ?)*[3/4]
yopC (pol. III, zeta)*[1]	*yorS* (pol. III, alpha)*	*yosH* (pol. III, alpha)*	*yozK* (pol. IV, DinB)[3/4]
uvrX (pol. IV, DinB)[1/2]	*yorL* (pol. III, alpha)	*yorS* (pol. III, alpha)*	*yosL* (pol. III, beta)*[3/4]
yqjW (pol. IV, DinB)[1/2]	*yomH* (pol. III, gamma, tau)*	*yorL* (pol. III, alpha)	*yosH* (pol. III, alpha)*[3/4]
yqjH (pol. IV, DinB)[1/2]	*uvrX* (pol. IV, DinB)	*yorC* (pol. III, gamma, tau)*	*yorS* (pol. III, alpha)*[3/4]
	yqjW (pol. IV, DinB)	*yomH* (pol. III, gamma, tau)*	*yorL* (pol. III, alpha)[3/4]
	yqjH (pol. IV, DinB)	*uvrX* (pol. IV, DinB)	*yoqC* (pol. III, delta)*[3/4]
		ypuA (pol. V, UmuC)*	*yopC* (pol. III, zeta)*[3]
		yqjW (pol. IV, DinB)	*yonI* (pol. I, PolA)*[3/4]
		yxlH (pol. IV, DinB)	*yomH* (pol. III, gamma, tau)*[3/4]
			uvrX (pol. IV, DinB)[3/4]
			yqjW (pol. IV, DinB)[3/4]
			yqjH (pol. IV, DinB)[3/4]

Genes are listed by map position. The underlined genes are part of the prophage SPβ chromosome in *B. subtilis* 168 genome. (http://www.genome.jp/dbget-bin/get_pathway?org_name=bsu&mapno=03030); DNA polymerase – *B. subtilis* 168 (KEGG PATHWAY Database; Kanehisa and Goto, 2000); *DNA polymerase with an Expect (E) value above 1e-03 (similarities to sequence alignments for homologs). [1/2] Ionizing radiation exposure to [1] X-ray and heavy ions ([2] Fe 500 MeV/n) irradiation. [3/4] High vacuum exposure for [1] 30 d and [2] 450 d (10^{-7} Pa).

The majority of the up-regulated DNA polymerases belong either to DNA polymerases (III) or (IV, DinB). Because DinB (DNA polymerase IV) has been shown to be involved in at least one aspect of stationary-phase mutagenesis in *E. coli* (Sung et al., 2003), several members of the Y family (i.e. *uvrX*, *yqjH* and *yqjW*) were investigated for their role in the mutagenesis in stationary-growth phase *B. subtilis*. UvrX is a UV damage repair protein that is assumed to be part of the endogenous bacteriophage SPβ (Kunst et al., 1997), and it has high identity to the DinB protein. Two further DNA polymerases IV (DinB proteins), *yqjH* and *yqjW*, were recently tested on their influence in the UV resistance of *B. subtilis*, it was found that both are products involved in UV-induced mutagenesis and

lacking of *yqjH* lead to dramatic decrease in the cell survival after UV exposure (Sung et al., 2003). Interestingly, *uvrX* was significantly induced by all treatments, which suggests that *uvrX* acts more as a (unspecific) DinB protein than as a specific UV damage repair enzyme, as expected by SubtiList-server (Moszer et al., 2002). Further on, it is important to note that the *yqjW* gene is part of an operon (*yqjWXYZyqkABC*), which includes genes involved in stress response. For example, *yqkB* (oxidoreductase) and *yqjZ* (monooxygenase), is part of the DNA damage-inducible SOS regulon of *B. subtilis* (Permina et al., 2002; Au et al., 2005). According to Yasbin et al. (1993) genes of the SOS regulon may also be induced in the absence of a functional RecA protein. This would explain the significantly high induction levels of several RecA-depending genes observed in this work. Duigou et al. (2005) reported that the DNA polymerase I (*polA*; the major DNA polymerase I, EC 2.7.7.7) acts in translesion synthesis mediated by the Y-polymerases (Pol Y1 *yqjH* and Pol Y2 *yqjW*) in *B. subtilis*. They suggest that Y-polymerases carry the synthesis across the lesion, and DNA polymerase I takes over to extend the synthesis until the functional replisome resumes DNA replication. Furthermore, they show that Pol I is linked to several key enzymes, e.g. DNA exonucleases, of the replication machinery in *B. subtilis*. This key role of Pol I in the translesion synthesis (TLS) uncovers a new function of the A-family DNA polymerases, e.g. *polA*, in the overall DNA repair. Kornberg and Baker (1992) showed that in *E. coli* the A-family DNA polymerase I plays an essential role in filling DNA gaps that arise during DNA replication, recombination and repair. However, the (potential) threshold of the (remaining) DNA damage, the nature and level of these lesion(s) in TLS during germination and outgrowth remain to be investigated. In short, the involvement of type IV DNA polymerases (DinB-like) (e.g. *uvrX*, *yqjH* and *yqjW*) in DNA repair ensures that DNA replication proceeds - even under circumstances of increased mutagenesis - by replication over typical DNA lesions e.g. DNA photoproducts and AP sites. Hence, TLS may be appreciated as a major cellular opportunity to assure survival of a wide population of cells (Dubnau and Losick, 2006).

In 2006, Bejerano-Sagie et al. (2006) reported of a specific DNA integrity scanning protein, called DisA (former YacK) involved in sensing of DNA lesions in *B. subtilis* entering the developmental process of sporulation. DisA is a non-specific DNA binding protein that forms a single focus, which moves rapidly within the bacterial cell, pausing at sites of DNA damage. DisA is focused on scanning along the chromosomes searching for lesions (Bejerano-Sagie et al., 2006; Boyle, 2006). Upon

encountering a lesion, DisA induces a cellular response that culminates in a temporary block in sporulation by affecting the phosphorylation in Spo0a. Interestingly, the *yacK* (*disA*) gene is part of a large operon (*ctsRmcsABclpCradAyacKLMNgltX*), including genes involved in the stress and radiation response e.g. *clpC* (stress response-related ATPase), *radA* (DNA repair protein homolog) and *yacL* (a predicted deoxyribonuclease) (Kunst et al., 1997). In this work, *yacK* (*disA*) was found to be up-regulated in weak, but significant levels in the transcripts after all stress treatments. This fact leads to the assumption that DisA is also involved in a DNA-damage checkpoint, which is active at the onset of the first genome replication (at 60 min after initiation of spore germination, according to Siccardi et al., 1977). The utilization of such a DNA-damage-scanning protein during germination would have essential advantages by ensuring an error-free genome replication from the (single) spore chromosome copy. It may be seen as an evolutionary strategy for providing an equal DNA copy transfer to all further isogenic offspring. Such an equal distribution of one (identical) genome version in the population may ensure the survival of the specific genotype if the population dynamics shift e.g. by the appearance of other *B. subtilis* subspecies. To approve this assumption several questions have to be answered: (i) What activates DisA during spore germination? (ii) What type of damage will be detected and/or be ignored? (iii) Is there a threshold of DNA damage for activating and/or blocking the function of DisA? (iv) Is there an increase in mutagenesis in germinating DisA-deficient spores? (v) Is there a coordination (programmed cascade) of NHEJ (based on its putative disadvantages) and DisA to ensure genomic integrity? and (vi) What is the role DisA in the general stress response of vegetative cells and its putative σ^B-regulation (according to Petersohn et al., 1999a; 1999b)?

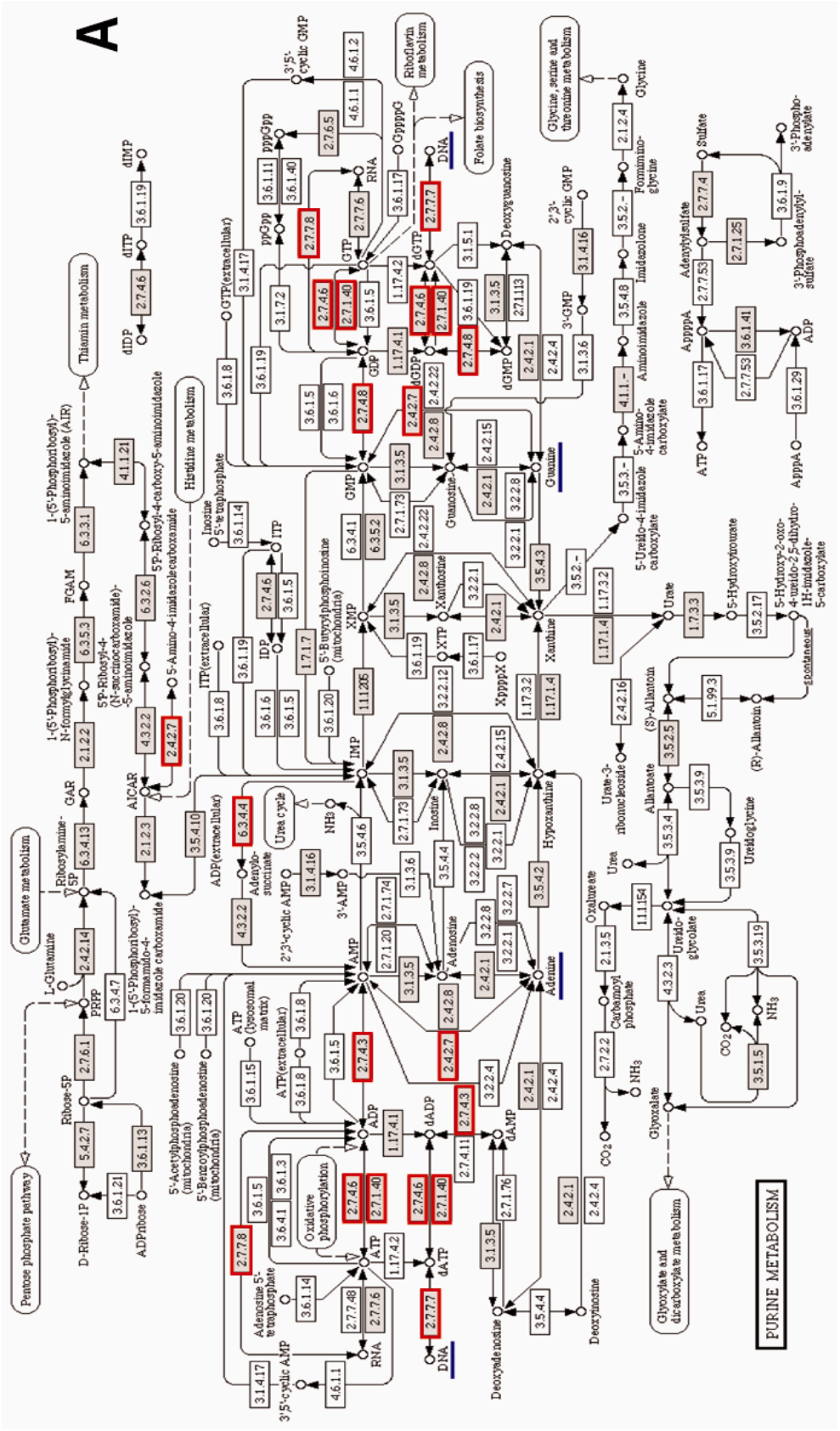
Communication between DNA replication, repair and cell cycle progression is imperative to maintain genomic stability (Sancar et al., 2004). To ensure completion of DNA repair before initiation of cell division significant changes in the transcript levels of cell division and DNA repair genes may lead to an interim pause in cell development. Whitehead et al. (2006) found that deletions of central enzymes in the pyrimidine metabolism significantly reduced the resistance of *Halobacterium salinarum* to γ-radiation.

In this work, it was found that in response to DNA damage-inducible treatments, such as UV or ionizing radiation, of the spores many genes involved in the metabolism of macromolecular precursors

of DNA were up-regulated during germination. These up-regulated nucleotide biosynthesis genes included several nucleoside/nucleotide kinases (e.g. *adk*, *ndk*, *tmk*) and those involved in purine (e.g. *purK*, *purR*) or pyrimidine (e.g. *pyrG*, *pyrR*) biosynthesis. Their activation indicates an increased *de novo* synthesis of nucleotides, a phenomenon consistent with damage responses observed in other prokayotes, such as *Deinococcus radiodurans* (Lui et al., 2003) or *Halobacterium salinarum* (Whitehead et al., 2006), as well as in higher eukaryotes i.e. human cell lines (Rieger and Chu, 2004). Increased nucleotide production may be necessary for different purposes: (i) to accommodate increases in transcription and (ii) for DNA replication and repair by replacing (damaged) nucleotides and/or synthesizing new ones. An increased nucleotide pool would also serve to enhanced ribosome genesis (Whitehead et al., 2006). The up-regulated genes (including their Enzyme Commission numbers) involved in the nucleotide metabolism (new synthesis) caused by DNA damage during spore germination are shown in Table 4-5. Their function in the purine or pyrimidine metabolism of *B. subtilis* are highlighted in Fig. 4-3.

Table 4-5 Genes involved in purine or pyrimidine biosynthesis, that were induced during germination of spores of *B. subtilis* treated with DNA damaging agents.

Ionizing radiation exposure: [1]X-rays/[2]Heavy ions (Fe 500 Me/V)	UV-A irradiation	UV-C irradiation	High vacuum [3]30 and [4]450 d exposure
cdd (EC 3.5.4.5)[1/2]	*aadK* (EC 2.7.7.-)	*adk* (EC 2.7.4.3)	*adk* (EC 2.7.4.3)[3]
dnaG (EC 2.7.7.-)[1/2]	*adk* (EC 2.7.4.3)	*cdd* (EC 3.5.4.5)	*dnaX* (EC 2.7.7.7)[3/4]
gmk (EC 2.7.4.8)[1]	*dnaN* (EC 2.7.7.7)	*dnaG* (EC 2.7.7.-)	*gmk* (EC 2.7.4.8)[3/4]
ndk (EC 2.7.4.6)[1/2]	*gmk* (EC 2.7.4.8)	*gmk* (EC 2.7.4.8)	*ndk* (EC 2.7.4.6)[3/4]
polC (EC 2.7.7.7)[1/2]	*ndk* (EC 2.7.4.6)	*ndk* (EC 2.7.4.6)	*pyrH* (EC 2.7.4.-)[3]
pyk (EC 2.7.1.40)[1/2]	*pnpA* (EC 2.7.7.8)	*purA* (EC 6.3.4.4)	*trxB* (EC 1.8.1.9)[3/4]
pyrG (EC 6.3.4.2)[1/2]	*polC* (EC 2.7.7.7)	*pyrG* (EC 6.3.4.2)	
pyrH (EC 2.7.4.-)[1]	*pyrG* (EC 6.3.4.2)	*pyrH* (EC 2.7.4.-)	
tmk (EC 2.7.4.9)[1/2]	*tmk* (EC 2.7.4.9)		
udk (EC 2.7.1.48)[1]	*udk* (EC 2.7.1.48)		
xpt (EC 2.4.2.7)[1/2]			


A
PURINE METABOLISM
Pentose phosphate pathway
Glutamate metabolism
Thiamin metabolism
Histidine metabolism
Folate biosynthesis
Riboflavin metabolism
Glycine, serine and threonine metabolism
Glyoxylate and dicarboxylate metabolism
Oxidative phosphorylation
Urea cycle
Ribose-5P
D-Ribose-1P
ADPribose
PRPP
L-Glutamine
GAR
FGAM
AICAR
IMP
AMP
ADP
ATP
GMP
GDP
GTP
XMP
XTP
Xanthine
Guanine
Adenine
Hypoxanthine
Inosine
Guanosine
Adenosine
Deoxyinosine
Deoxyguanosine
Deoxyadenosine
dAMP
dADP
dATP
dGMP
dGDP
dGTP
dIMP
dIDP
dITP
RNA
DNA
Urate
Allantoin
Urea
Glyoxylate
Oxaluric acid
Carbamoyl phosphate
Ureidoglycolate
Ureidoglycine
Glycine
Sulfate
Adenylylsulfate
3'-Phosphoadenylate
3'-Phosphoadenylyl-sulfate
5-Hydroxyurate
5-Hydroxy-2-oxo-4-ureido-2,5-dihydro-1H-imidazole-5-carboxylate
5-Ureido-4-imidazole carboxylate
5-Amino-4-imidazole carboxylate
Aminoimidazole
Imidazolone
Formimino-glycine
Urate-3-ribonucleoside
(S)-Allantoin
(R)-Allantoin
Oxidative phosphorylation
Adenylo-succinate
2',3'-cyclic AMP
3'-AMP
3',5'-cyclic AMP
2',3'-cyclic GMP
3'-GMP
3',5'-cyclic GMP
ppGpp
pppGpp
GppppG
Urea
NH3
CO2
Glycine

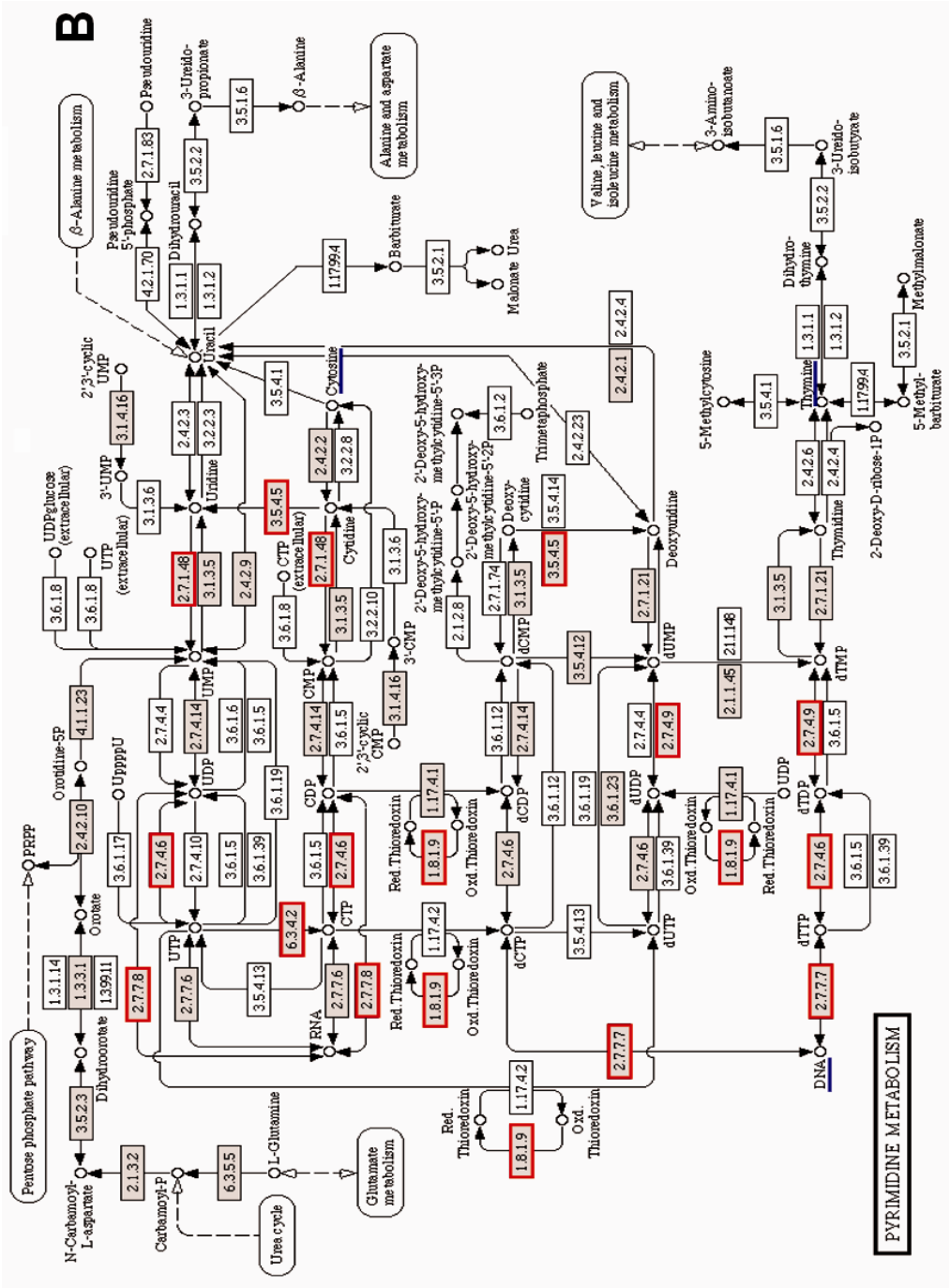


Fig. 4-3 Purine (**A**) and pyrimidine (**B**) metabolism of *B. subtilis* 168 (according to the nucleotide metabolism of the KEGG PATHWAY database; Kanehisa and Goto, 2000); Red-marked boxes show the up-regulated genes in response to UV, ionizing radiation and high vacuum exposure after 60 min germination. Blue-underlined highlight final key molecules, i.e. DNA and purine/pyrimidine nucleotides. Grey-filled boxes present known enzyme involved in the nucleotide synthesis, white-filled putative and/or yet unknown functional members of this pathway.

Mainly transcripts involved in completion of nucleotides, e.g. *ndk* EC 2.7.4.6 (that catalyzes DNA-template-directed extension of the 3'- end of a DNA strand by one nucleotide at a time), are significantly up-regulated during biosynthesis, leading to the assumption that at 60 min germination, a time point when the first genome replication begins or is initiated, new nucleotides are needed to replace damaged nucleotide and to ensure a successful DNA replication of the second genome. Due to the fact, that the nucleic acid synthesis in *B. subtilis* is a complex mechanism, which involves over 90 genes, further studies are needed on the role of genes involved in the purine and pyrimidine metabolism in the overall spore resistance. Further on, the fate of the damaged (DNA) nucleotides, especially the possible cellular protection of preventing the incorporation of damaged nucleotides in the DNA, the putative recycling of modified (damaged) nucleotides by e.g. degradation and/or exclusion during spore germination and outgrowth remain to be determined.

All bacterial cells are equipped with genetic programs allowing adaptation to a variety of environmental stresses (reviewed in Zellmeier et al., 2005). These programs result in the transient expression of a subset of genes to cope with the stressful situation. *B. subtilis* codes for a total of 16 alternative sigma factors participating in the sporulation process (σ^H, σ^E, σ^F, σ^G, and σ^K; Errington, 1993; Haldenwang, 1995), in chemotaxis, motility, and production of autolysins (σ^D), amino acids catabolism (σ^L) and in the general stress response (σ^B; Petersohn et al., 1999a; 1999b). A further subfamily of sigma factors is involved in responses to environmental challenges, the so-called extracytoplasmic function subfamily (ECF sigma factors). A total of seven ECF sigma factors in *B. subtilis* (σ^M, σ^V, σ^W, σ^X, σ^Y, σ^{YlaC}, and σ^Z) were defined from sequence comparison (Lonetto et al., 1994). In this work, the ECF sigma factor σ^W was found to be significantly up-regulated after 60 min germination as response to sporicidal (DNA damage-inducible) treatments. Cao et al. (2002) reported that genes controlled by σ^W can be induced by detoxification response, alkaline shock, high concentrations of sodium chloride, phage infection and cell wall antibiotics. In 2003, Asai et al. (2003) identified target gene candidates of σ^W by DNA microarray analysis. They identified several DNA repair, replication and nucleotide metabolism-related genes: *ligA*, *murG*, *pyrR*, *mutL*, *dinG*, *udk*, *yrvE*, *radC* and *ssb* under σ^W control (Asai et al., 2003). Interestingly, these were also found to be strongly induced during germination of UV, ionizing radiation or high vacuum exposed spores, as detected in this work. In the case of SigW, a possible DNA damage response sigma factor, further experiments are

awaiting for the delineation of its exact role in radiation- and desiccation-stress response and recovery during germination, complementary to previous studies performed with σ^B-depending stress genes in vegetative cells (Haldenwang, 1995; Petersohn et al. 2001).

4.4.3 RecA-independent inducible genes during spore germination

In addition to the wild-type spores, the transcriptional responses of *recA*-deficient spores (treated *vs.* untreated) to X-ray, accelerated heavy ions, mono- or polychromatic UV irradiation, as well as high vacuum were determined after 60 min germination. The aim was to receive information on the induction of putative RecA independent repair systems. RecA is known to be a major component in the initiation of SOS response through its activation by DNA damage, probably by binding to single-stranded DNA arising as a consequence of the damage (Little, 1991; Friedberg, 1995). Following DNA damage, the activated RecA protein stimulates the autocatalytic cleavage of the LexA repressor protein, which binds to a sequence termed the SOS box upstream of the genes (Brent and Ptashne, 1981; Little et al., 1981). The resulting fragments of LexA do not efficiently bind to the SOS box (Bertrand-Burggraf et al., 1987), resulting in increased transcription of the genes it regulates. This process is dependent on the presence of functional RecA, and in a *recA*-mutant strain genes regulated by LexA are no longer DNA damage inducible (Weisemann et al., 1984). This mechanism of LexA regulation has been best defined in *E. coli* (Rand et al., 2003); however, in many species, such as *B. subtilis* (Lovett et al., 1988; Gassel and Alonso, 1989), the response is regulated by homologues of the *E. coli* repressor protein LexA (Eisen and Hanawalt, 1999). In *B. subtilis*, some genes belonging to the SOS regulon can also be induced, when the cell enters the competent state; however this is a separate mechanism (Lovett et al., 1989; Haijema et al., 1996; Hamoen et al., 2001).

A RecA independent induction of repair genes in response to DNA damage is relatively rare, but has now been described for a number of bacterial species (Campoy et al., 2002, 2003), including *E. coli* (Gibert et al., 1990; Kleinsteuber and Quinones, 1995) and *Mycobacterium tuberculosis* (Rauch et al., 1996; Davis et al., 2002; Rand et al., 2003). In this work, several genes were found to be significantly up-regulated in the *recA*-deficient mutant in response to DNA damaging treatments (Table 4-6) (for detailed information on gene activation see Tables 3-8, 3-13, 3-20 and 3-22), although for each GO the number of induced genes was much lower than in the wild-type strain. Several transcripts (e.g. *yppQ*, *ndk*, *yqjH*, *ywqH*, *ssb*) were found to be up-regulated after treatment with UV and ionizing radiation as

well as after high vacuum exposure, leading to the suggestion that these genes are general stress response-induced *recA*-independent genes (e.g. *yppQ*) or coding for DNA restoration e.g. DNA strand binding/stabilization proteins (e.g. *ssb*, *ywqH*). Another gene, *ywjD*, a predicted UV-endonuclease, was activated only after UV radiation. *ywqH*, a putative single-strand DNA-binding protein, was found to be activated after DNA double-strand break inducing treatments (i.e. ionizing radiation or high vacuum exposure).

These results are first indications of *recA*-independent up-regulation of repair genes during spore germination in response to spore DNA damage. However, the precise gene activation and regulation of those genes (i.e. repair mechanism) as well as the nature of the inducing and repairable damage remain to be investigated, which was beyond the scope of this work. Further studies are required to investigate whether those *recA*-independent inducible genes represent an alternative (or general) mechanism for the induction of RecA-independent DNA repair pathways as potential "by-pass" adaptation during stress response of germinating spores.

Table 4-6 Genes highly induced during germination of irradiated or vacuum-exposed *recA*-deficient spores (with at least two entries)

Gene*	GO	(predicted) Function / Operon	UV radiation	Ionizing radiation	High vacuum
ykoQ	1	unknown; similar to DNA repair exonuclease / phosphoesterase [*B. cereus* E33L, E value 6e-91] / *ykoQ*	C, A	X	
ymaD	6	unknown; similar to organic hydroperoxide resistance protein OsmC [*Legionella pneumophila* subsp. *pneumophila* str. Philadelphia 1, E value 2e-07] / *ymaCD*	A	X, Fe	
ymaB	1	unknown; similar to phosphoesterase [*B. clausii* KSM-K16, E value 7e-60]; MutT/nudix family protein [*Staphylococcus epidermidis* RP62A, E value 9e-34] / *ymaAnrdEFymaB*	C, A	X	
yocH	7.1	unknown; similar to cell wall-binding protein / N-acetylmuramoyl-L-alanine amidase [*Oceanobacillus iheyensis* HTE831, E value 5e-46/3e-11] / *yocIH*	C	X	
yppQ	6	unknown; similar to methionine sulfoxide reductase B [*B. subtilis* 168, E value 4e-82] / *msrAyppQ*	A	X, Fe	V
ndk	5	nucleoside diphosphate kinase / *hepSmenHhepTndk*	A	X	V
yqjH	1	unknown; similar to DNA polymerase IV [*B.* sp. NRRL B-14911, E value 1e-130] / *yqjHI*		X, Fe	V

radC	1	probable DNA repair protein / *mafradCmreBCDminCD*	C, A	X, Fe	
ywqH	3	unknown; similar to single-strand DNA-binding protein [*B. licheniformis* ATCC 14580, E value 2e-37] / *ywqGHIJKL*		X, Fe	V
ywjD	1	unknown; similar to UV-endonuclease (UvsE / Uve1 / UvdE family) [*B. thuringiensis* serovar *israelensis* ATCC 35646, E value 1e-109] / *ywjEDC*	C, A		
ssb	3	single-strand DNA-binding protein / *yyaEFrpsFssbrpsR*	C, A	X, Fe	V

* Genes are listed by map position. For details on the gene function and ontology, see Table 3-8/3-9, 3-13/3-14, 3-20/3-21 and 3-22/3-23. C = UV-C: 254 nm; A = UV-A: 320 - 400 nm; X = X-rays (150 keV/19 mA); Fe = Iron-ions (Fe 500 MeV/n, LET 200 keV/µm); V = high vacuum exposure (10^{-7} Pa); GO = Gene ontology group.

4.4.4 The NHEJ system

As mentioned before the main damage induced in spores by ionizing radiation are DNA strand breaks (SSB and DSB) (Hutchinson, 1985; Nicastro et al., 2002). Therefore, NHEJ as putative DNA strand breaks repair mechanisms (Fig. 4-4) might be an important pathway during germination of spores treated with agents that induce DNA strand breaks. In NHEJ, the first step involves the binding of the Ku complex to the two DSB ends (*ykoV*). The next proteins to be recruited to the complex are those that lead to resection of the ends of a DSB. The final set of steps in NHEJ leads to direct joining of the two ends by a DNA ligase (*ykoU*) that restores the integrity of the DNA.

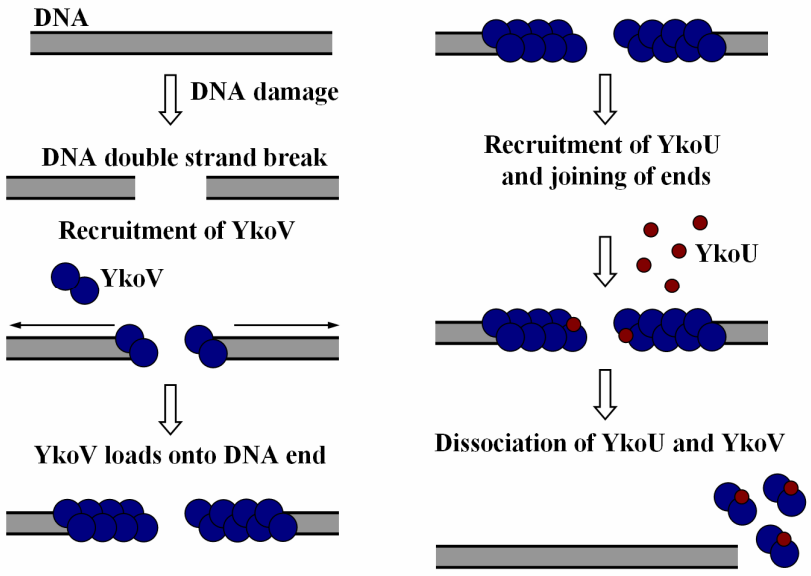


Fig. 4-4 Biochemical model for non-homologous end-joining (NHEJ) in *B. subtilis* 168 (adapted from Wilson et al., 2003; Pitcher et al., 2007).

It is important to note that NHEJ does not require more than one chromosome – as recombination-mediated repair (RR) does – and is therefore a promising candidate for DNA strand break repair induced in bacterial spores. This importance of NHEJ in the repair of DNA strand breaks induced in spores was tackled by using spores three different NHEJ-defective mutants (*ykoV, ykoU,* and the double mutant *ykoU ykoV*) and comparing their sensitivities with those of the wild-type counterparts. If was found that NHEJ-defective spores were more sensitive to X-rays (RF = 15 - 26 %) than wild-type spores, and even more sensitive than *recA*-deficient spores (RF = 35 %). This observation leads to the assumption that germinating spores more likely repair induced DNA DSB by NHEJ than by recombination-mediated repair (*recA*). However, RecA is also an important signaling molecule in the SOS response, via its protease activity on the Lex repressor (Miller et al., 1996; Au et al., 2005); lack of SOS induction in the *recA* mutant may explain its increase in sensitivity to X-rays and HZE particles. Included in the SOS regulon of genes induced by UV or mitomycin C are a number of operons encoding putative or unknown functions; some of these genes may encode proteins involved in repair of ionizing radiation damage, and may be targets for future exploration. The covalent rejoining of DNA ends at single-stranded or double-stranded DNA breaks is catalyzed by DNA ligases. Ligases catalyse the formation of phosphodiester bonds at nick sites between 3'-hydroxyl and 5'-phosphate termini in double-stranded DNA. However, further studies of the nature of the DNA damage induced in spores by ionizing radiation (directly or indirectly caused) are needed. In any case, the discovery of the *ykoVU*-operon has brought markedly new insights in the understanding of the resistance properties of bacterial spores (Weller et al., 2002; Wang et al., 2006; Pitcher et al., 2007).

Spores carrying the *ykoU, ykoV,* or *ykoU ykoV* mutations were also significantly more sensitive to high vacuum exposure with RF values between 11 % and 15 % for those carrying a single mutation, and of 9 % for those carrying the double mutation. This extremely high sensitivity of NHEJ-deficient spores to high vacuum-induced desiccation, which even excelled that in response to X rays, is entirely consistent with the observation that exposure to high vacuum induced SSB and DSB formation in DNA (Dose et al., 1991; 1992). Thus, NHEJ is an extremely important determinant of spore survival to the high vacuum prevailing in space, a critical prerequisite for viable interplanetary transport of spores by natural processes or by human spaceflight activities (reviewed in Horneck, 1993; Nicholson et al., 2000; 2005). Several above described mechanisms and strategies can (possibly) explain the high

survival shock pressure-treated spores: the utilization of NHEJ as DNA strand break repair mechanism during spore germination and the stabilization of the DNA structure by SASPs as DNA repair and protection strategy, respectively.

Slieman and Nicholson (2000) report on DNA strand-break (SSB and DSB) induction in *B. subtilis* spore DNA by solar UV, putatively generated by ROS interaction with spore DNA. In this thesis, the role of NHEJ in the spore resistance to mono- and polychromatic UV was analyzed. Spores deficient in NHEJ were significantly more sensitive to UV radiation. Whereas in response to UV-C radiation the RF values were quite high (55 - 60 % for the single mutants and about 50 % for the double mutant), which indicates a relatively low involvement of NHEJ, they gradually decreased when extending the wavelength range to UV-B and UV-A. One should not forget that SP is the overwhelming DNA damage of UV-C and this is effectively repaired in-*situ* by the SP lyase. Spores exposed to a simulated terrestrial UV spectrum, i.e. polychromatic UV radiation at wavelengths > 290 nm, showed slightly lower RF-values (50-60 % for the single mutants and about 40 % for the double mutant), although these values were not significantly different from those for monochromatic UV-C. The role of NHEJ became more obvious in response to UV-A radiation with RF values of 26 - 32 % for the single mutants and about 20 % for the double mutant. These data further support the notion that UV-A produces a substantial amount of strand breaks in the DNA of spores - through indirect mechanisms, and that NHEJ is a major DNA repair mechanisms during spore germination to ensure genome integrity. These observations as well as previous reports (Slieman and Nicholson, 2000; Moeller et al., 2007c) lead to the assumption that NHEJ is a major ecological mechanism to sustain the resistance of spores in natural habitats governed by extreme UV radiation exposure and extended desiccation. The involvement of NHEJ in responses to different stressful treatments of bacterial spores is summarized in Table 4-1.

The ligation of DNA strands during NHEJ is an energy-dependent process and ATP is required for the catalysis of the formation of a phosphodiester at the site of a single-strand break (SSB) in a duplex DNA. In this case, it is worthwhile to note that dormant spores do not contain detectable amounts of endogenous ATP; rather, high-energy phosphate is stored in the spore as a large depot of 3-phosphoglyceric acid (3-PGA). In the first minutes of spore germination, 3-PGA is rapidly converted

to ATP by the lower branch of glycolysis (Setlow and Kornberg, 1970; Setlow et al., 2001). Thus the activity of the NHEJ pathway is dependent upon reactivation of ATP generation during spore germination, since *ykoU* is supposed to encode an ATP-depending DNA ligase (Weller et al., 2002).

Wang et al. (2006) reported recently on the role of NHEJ (alone or coupled with the influence of SASPs) in spore resistance to dry heat, which is a potential inducer for DNA strand breaks in spore DNA by possibly generating apurinic and apyriminidinic sites. They found out that both, NHEJ- and major SASPs-deficient (and the respective double-mutant) spores, were significantly more sensitive to dry heat than wild-type spores. This experiment (and the results) may be considered as a rather good simulation of the effects of thermal spikes or shock waves caused e.g., by penetrating high energy charged particles (heavy ions) or in the case of meteorite impacts. The observations lead to the assumption that, besides SASPs, NHEJ is an important factor in spore resistance to cope with DNA strand breaks inducing treatments e.g. ionizing radiation, high velocity impacts, or extreme desiccation (Dose et al., 1991; 1992; Weisbrod et al., 1992; Potts, 1994).

During the analysis of the role of NHEJ in the overall spore resistance and due to some structural/morphological limitations (e.g. single genome per spore, metabolic inactive) several potential disadvantages can be hypothesized caused by deficiency and disruption of the NHEJ-complex, mainly with regard to mutagenesis and attenuation of the genome integrity, which may have a direct impact on microbial evolution. If, for instance, ionizing radiation has damaged the ends of a DNA DSB, then it might be predicted that repair (or removal) of two contiguous damaged ends followed by their relegation would lead to a variety of point mutations or small deletions. Alternatively, if two non-contiguous ends of different DNA DSB have been joined, then this could lead to gross rearrangements of the genomes and creation of new gene combinations at the junctions. However, these assumptions remain to be determined.

The above results raise the question of the potential role of *ykoW* in the (actual correct) *ykoWVU* operon in NHEJ (Kunst et al., 1997). According to the SubtiList-database (http://genolist.pasteur.fr/SubtiList), the putative *ykoW* product is similar to either sensory box proteins and/or diguanylate cyclase/phosphodiesterases potentially involved in DNA repair (Moszer et al., 2002). In this regard it is interesting to note that Mun et al. (1994) reported that non-homologous ends

were removed by a DNA phosphodiesterase to eliminate radiolytic products (e.g. thymine glycol adducts) at broken DNA ends in *Deinococcus radiodurans*. Induction of oxidative DNA damage e.g. thymine glycol adducts were detected in significant level in spores exposed to ionizing radiation. In addition, YkoV ligases themselves from a number of bacteria are reported to encode DNA polymerase and DNA end-processing activities (Bowater and Doherty, 2006). These observations point to the intriguing possibility that the *ykoW* product may also function to prepare DSB ends for resection, a possibility that is currently being addressed by several researchers.

4.5 Key transcriptional events to ensure DNA restoration during spore germination and outgrowth

DNA damage accumulated in bacterial spores and perturbations in DNA replication induce global transcriptional responses that enable germinating spores to repair the induced damage and hence to survive (by error-free and undisturbed outgrowth). Several DNA protection strategies, e.g. SASPs, and DNA repair mechanisms e.g. SP lyase and NHEJ (under σ^G-regulation) are already synthesized during sporulation to ensure DNA repair directly after activation during germination. SP lyase specifically repairs UV-induced SP dimers by in-*situ* monomerization into the two original thymine residues. As outlined in 4.4.4, NHEJ is especially prone to repair SSB and DSB of DNA, which may be caused directly or indirectly by several stress treatments, such as radiation, extreme desiccation, high pressures or high temperatures. Besides those repair systems that are already present in the spore and may, as in the case of NHEJ, need only re-activation of ATP to succeed, a great number of genes involved in DNA damage repair need to be up-regulated during germination of the spores. Deficiencies in those genes, involved in DNA damage repair, lead to a significant decrease in the spore survival in response to UV, ionizing radiation or extreme desiccation, depending on the type of damage caused by the treatment and the specificity of the respective repair system under investigation.

It was shown in this work that many DNA damage-inducible (*dinB*, *lexA*, *recA* and *tagC,* former *dinC*) genes and DNA damage-responsive (e.g., *recF*, *recG*, *recO*, *recU*) genes were induced in the spores after 60 min germination regardless of the DNA damaging treatment the spores had experienced. In general the genomic responses of wild-type *B. subtilis* spores to the different treatments, such as UV-C, UV-A, X-rays, accelerated HZE particle irradiation or extreme desiccation, measured after 1 h of

germination recovery, were quite similar - at least to a substantial extent. Those responses included the induction of systems involved in DNA modification (i.e. repair, recombination, replication and packaging), in nucleotide replacement (i.e. binding, synthesis and nucleotide acid metabolism) as well as in adaptation (i.e. stress response). These results lead to the assumption that there might be a general "built-in" transcriptional germination program for key transcriptional events, to activate checkpoints for ensuring DNA integrity (Fig. 4-5).

The numbers in Fig. 4-5 refer to the number of genes of functional groups that were highly expressed during germination of spores exposed to various DNA damaging treatments (identified by the color code). The major DNA repair pathways are also identified, those already synthesized during sporulation (underlined) and those that need to be up-regulated during germination.

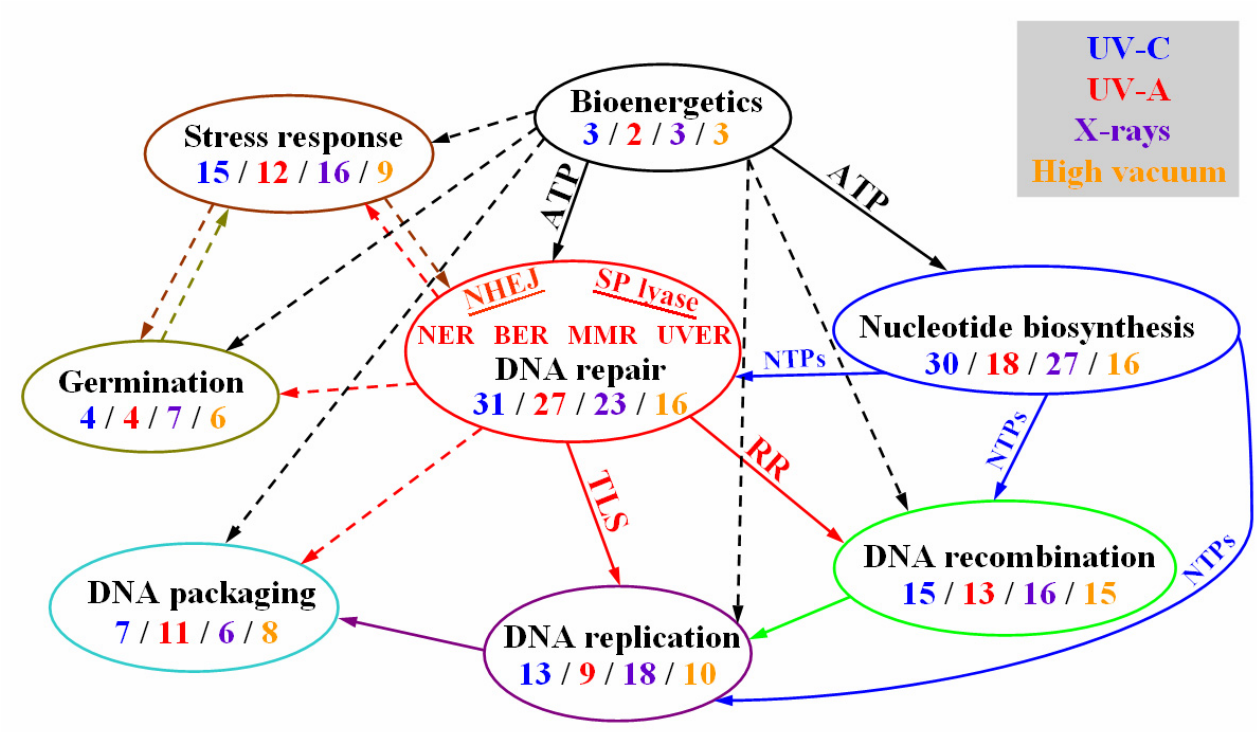


Fig. 4-5 Scheme of a "built-in" transcriptional germination program of bacterial spores, representing key transcriptional events to ensure DNA restoration for spore germination and outgrowth. Used abbreviations (if not explained before): ATP: adenosine triphosphate, NTPs: nucleotide triphosphates, MMR: mismatch repair, UVER: UV endonuclease, BER: base excision repair. Solid lines outline the direct interactions and dashed lines the indirect relations of the respective gene ontology groups to each other in framework of the DNA restoration program during spore germination.

A similar program has recently been proposed by Keijser et al. (2007); however, in contrary to the findings of Keijser et al. (2007), DNA-repair related sporulation transcripts were not found to be up-regulated in germinating spores after stress treatment. An example is the SP lyase encoding gene *splB* that was not up-regulated during germination of UV-irradiated spores.

The current and previous studies have revealed that there are two major strategies to constitute the uniqueness in spore resistance (Fig. 4-6):

(i) "forward" spore protection (Fig. 4-6: A, B, C) by the assembly of spore-specific features e.g. Ca-DPA-complex, dehydration, DNA A-conformation, spore coat layers, spore core minerals, DNA binding by SASPs and the synthesis of specific DNA repair enzymes, such as SP lyase and the NHEJ system during sporulation, and

(ii) "backward" spore repair (Fig. 4-6: D, E) during germination and outgrowth of damage accumulated in the spore state (in spite of the "forward" protection mechanisms). They included reactivation of the ATP- and macromolecules-biosynthesis, (universal pathways or spore specific), and a widespread induction of systems involved in DNA modification (i.e. repair, recombination, replication and packaging), in nucleotide replacement (i.e. binding, synthesis and nucleotide acid metabolism) and in adaptation (i.e. stress response). This general scheme of "backward" spore repair fits best in a scheme of a "built-in" transcriptional germination program of bacterial spores, which represents one of the clues to the understanding of the unique resistance of bacterial spores to environmental stresses. The present work has especially contributed to the understanding of the involvement of several of these features, e.g., α/β-type SASPs, NHEJ-complex and SP lyase in the general spore survivability to different radiation and pressure treatments (Table 4-1).

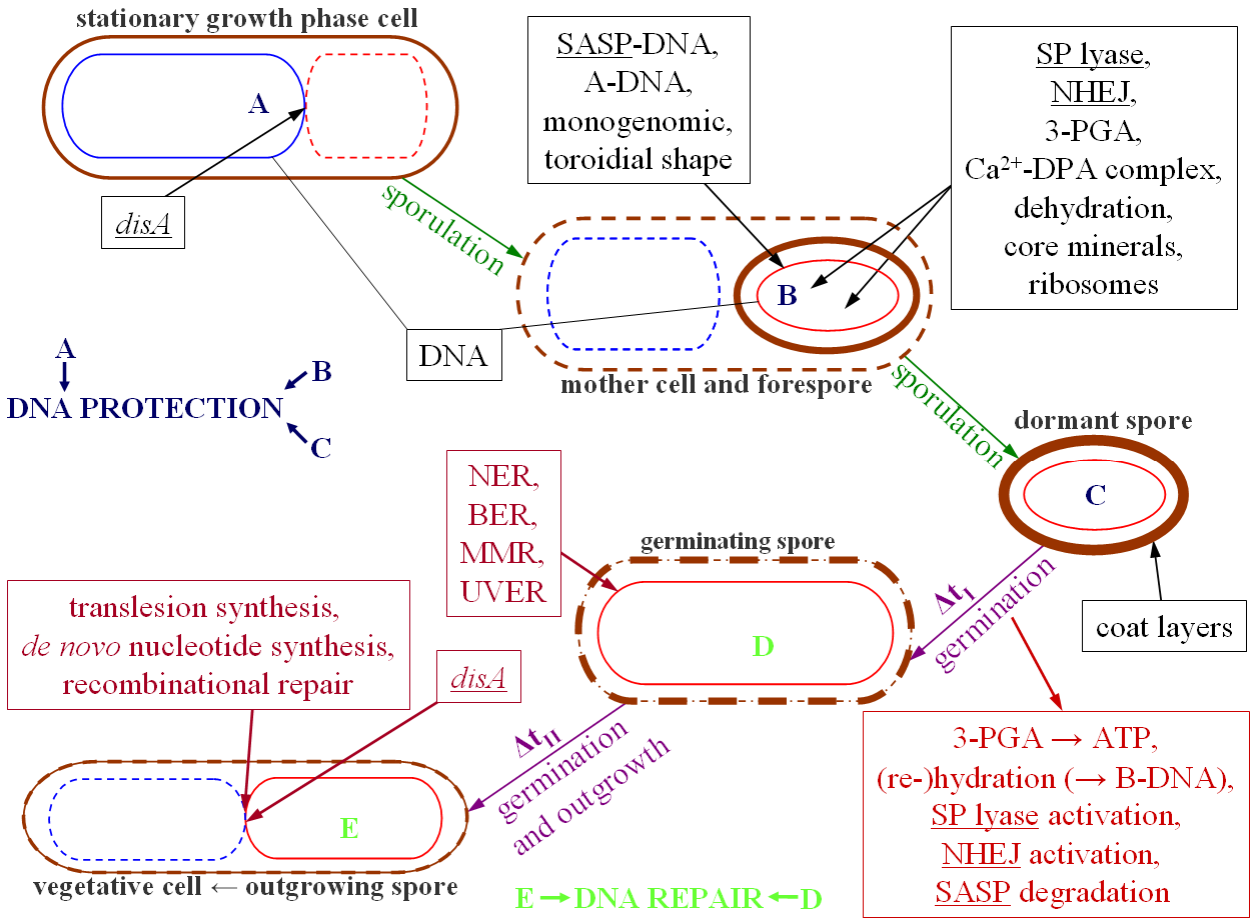


Fig. 4-6 Interrelationship of the DNA protection strategies (A-C) and repair mechanisms (D, E) of sporulating, germinating and outgrowing *B. subtilis* spores. Data and information were generated in this work (see chapter 3 and 4) and derivated from references (see chapter 6). Used abbreviations (if not explained before): *disA* DNA integrity scanning protein, 3-PGA 3-phosphoglyceric acid. Solid lines outline the existing DNA and/or vegetative cell wall/spore coat layers and dashed lines the degrading/lysing (for the mother cell) and forming/new synthesizing (for the dormant, germinating and outgrowing spores) DNA and/or cell wall/spore coat layers.

The present understanding of the mechanism involved in spore resistance is as follows: The degree of spore resistance is dependent on a number of factors, with different factors having differing degrees of importance for a particular treatment. Major factors involved in spore resistance include the relative dehydration of the spore core, the mineralization of the spore core, the relative impermeability of the spore core to potentially DNA-damaging agents, the spore coat layers, the Calcium-DPA-complex, the protection of spore DNA by binding of SASPs, the structure and topology of the spore DNA, and DNA repair during germination. Essential DNA mechanisms for repairing spore DNA damage, e.g. NHEJ

and SP lyase, have been identified and characterized. During spore germination, before vegetative growth is restored, ATP synthesis resumes, the DNA-complexing SAPSs are degraded by a protease (providing a source of amino acids for outgrowth) and accumulated DNA damage is repaired. In this thesis, the induction and/or repair of spore (DNA) damage were measured after UV, ionizing radiation and extreme desiccation and the transcriptional profile in response of DNA damage-inducible treatments after 1 h spore germination were determined. In this time-frame, when the first genome replication begins or is initiated, essential enzymes involved in the nucleotide metabolism (e.g. nucleoside/nucleotide kinase(s)), DNA replication (e.g. resolvase(s)/recombinase(s)), DNA damage sensing (e.g. *disA*), stress response (e.g. *ytkD*) and repair (e.g. *ywjD*) were found to be significantly up-regulated. This simultaneous analysis of transcriptional and biochemical changes during spore germination has provided additional functional information on the spore resistance to extreme environmental stressors such as UV radiation and extreme desiccation. These findings improve the understanding of spore-specific and general pathways necessary for DNA damage repair and germination, as well as the molecular basis for the unique resistance of dormant spores.

5. Summary

Aim: It is the aim of this thesis to provide further insights to the understanding of the mechanisms of the extremely high resistance of bacterial endospores to environmental stress factors.

Materials and Methods: Spores of the Gram-positive bacterium *Bacillus subtilis* 168 wild-type as well as descendents deficient either in SASPs or selected repair pathways, were exposed to the following terrestrial or simulated extraterrestrial stress conditions: UV radiation (UV-C at 254 nm, polychromatic UV-A at 320 - 400 nm), ionizing radiation (X-rays or accelerated heavy ions: He at 2.2 keV/µm, C at 12 keV/µm, Si at 50 keV/µm, Fe at 200 keV/µm), extreme desiccation (high vacuum at 10^{-7} Pa), or shock pressures in the range of 5 - 50 GPa, simulating a high velocity meteorite impact. A standardized experimental protocol for preparation and analyses was established to allow comparison of the responses of spores of different strains to the different stress parameters. The analyses included:

(i) Survival by the ability to form macroscopic visible colonies (CFA);

(ii) Spectrum of DNA base damage by HPLC-MS/MS (UV-induced bipyrimidine photoproducts, ionizing radiation induced oxidative damage);

(iii) Role of SASP in protecting the spore DNA by use of a SASP deficient mutant strain (*sspA sspB*);

(iv) Mutational specificity (Rif^r mutations induced by ionizing radiation or vacuum in *rpoB* by use of PCR, phenotypic Spo^- mutants after shock pressure treatment);

(v) Role of different repair pathways (especially SP-lyase, recombination-mediated repair, nucleotide excision repair, non-homologous end-joining system) by comparing the responses of repair deficient mutants (*recA* for recombination mediated repair, *splB* for SP specific repair, *splB uvrB* for SP specific repair in combination with NER, *recA splB uvrB* for all three pathways, *ykoV, ykoU,* and *ykoU ykoV* for the NHEJ system) with those of their wild-type counterparts;

(vi) cDNA microarray-mediated transcriptional profiling of (treated vs. untreated) wild-type or *recA* spores during germination;

(vii) Comparative validation of selected up-regulated genes involved DNA integrity restoration by RT-PCR.

Results: It was shown that all environmental stress parameters tested led to inactivation of the bacterial spores in a dose dependent manner. The spore DNA was the main sensitive target, for the different radiation qualities, but also for vacuum or high velocity shock pressure treatments. Among the spectrum of UV induced bipyrimidine photoproducts, which all linearly increased with the fluence (after UV-C as well as UV-A irradiation) SP was the predominant photoproduct. α/β-type SASPs deficient spores (*sspA sspB*) were more sensitive than the wild-type spores, (5-10 times with regard to UV-C or UV-A and 3 times with regard to X-rays or accelerated heavy ions). In those mutants SP was hardly – if at all – induced by UV-C or UV-A, however the induction rate of oxidative damage (8-oxodGua and ThdGly) by ionizing radiations was twice as high as in the wild-type spores. These observations further stress the major role of SAPSs in spore DNA protection by structural stabilization, but probably also as scavenger of radical oxygen species.

Mutations to Rif^r induced by ionizing radiations or by vacuum treatment were caused by base changes (43.3 % transitions and 46.7 % transversions) and were mostly (93 %) located in cluster I of the *rpoB* gene. They revealed four mutational hot spots. Six Rif^r mutants were first described in this work: 4 in cluster I, and 2 between cluster I and II. These observations provide further support to the assumption that vacuum treatment is mutagenic and affects the DNA of spores. Phenotypic Spo^- mutations, induced after hypervelocity shock pressure treatment of spores hint to a mutagenic effect also of high shock pressures.

During spore revitalization, DNA damage accumulated during the dormant spore stage is the subject of a variety of different repair systems. Deficiencies in repair genes led to a significant decrease in spore resistance to UV, ionizing radiation or extreme desiccation. Concerning UV-C or UV-A radiation, the resistance of spores, taken from the RF values (repair factor with regard to survival), decreased in the following order: wild-type > *recA* > *ykoV* or *ykoU* or *ykoU ykoV* > *splB* or *splB uvrB or recA splB uvrB*. A similar trend was observed for bipyrimidine photoproduct removal by the different mutants (not determined for the NHEJ deficient mutants). The repair efficiency of SP was remarkable decreased in the SP lyase deficient mutants, reaching 6 - 8 % of that in wild-type spores. These data confirm the overwhelming role SP lyase plays in spore UV resistance. Concerning ionizing radiations as well as in response to vacuum exposure the resistance of the spores decreased as follows: wild-type > *splB* > *recA* > *ykoV* or *ykoU* or *ykoU ykoV*. The repair efficiency of the NHEJ double mutant was reduced to 15 % after X-irradiation and to 9 % after vacuum treatment compared to the wild-type spores, indicating

both, (i) the important role this recently discovered NHEJ system plays in DNA repair of spores after those treatments, and (ii) DNA strand breakage being a critical damage caused by ionizing radiation as well as by vacuum treatment. Herewith NHEJ (encoded by *ykoU*, ATP-depending DNA ligase, and *ykoV*, bacterial Ku-protein) has shown to be a key strategy used during spore germination to repair DNA double strand breaks caused by terrestrial or extraterrestrial physical stress conditions.

To achieve detailed information of the global gene expression during germination of treated spores a methodology for the rapid and simple extraction of high-quality total RNA from dormant and germinating spores of *B. subtilis* was developed and used. The majority of the up-regulated transcripts were directly assigned to specific functional categories involved in radiation- or low pressure-induced damage repair, macromolecule biosynthesis, or stress response. Known and newly identified (DNA) damage-responsive genes ("*y*"-genes), regulating key DNA repair processes e.g. (nucleotide) excision repair, recombination mediated repair or (error-prone repair) translesion synthesis during spore germination were found to be significantly induced. After 60 min germination, when the first genome replication begins or is initiated, several new candidate genes for encoding structure-specific damage-inducible resolvase/recombinase (involved in recombinational repair; e.g. *yneB*), DNA damage sensing (e.g. *disA*), stress response (e.g. *ytkD*, involved in the oxidative DNA stress response, as potential 8-oxo-dGTPase), translesion synthesis (e.g. *yqjH*, a DinB-like Y1-DNA polymerase) and excision repair (e.g. *ywjD*, a putative *recA*-independent UV damage endonuclease) were identified. Much less, but still a significant number of genes were induced during germination of treated *recA* mutant spores, pointing to the possible existence of RecA independent damage inducible repair pathways.

In general, the individual transcriptional responses of *B. subtilis* 168 spores to treatments by either UV-C, UV-A, X-rays, accelerated heavy ions or extreme desiccation showed certain similarities. Those corresponding responses included the induction of systems involved in DNA modification (i.e. repair, recombination, replication and packaging), in nucleotide replacement (i.e. binding, synthesis and nucleotide acid metabolism) as well as in adaptation (i.e. stress response).

Conclusions: The similar – partly identical - responses of spores to various DNA damaging stresses point to the existence of a general "built-in"-transcriptional germination program in spores with key transcriptional events as checkpoints for ensuring DNA restoration and integrity. "Forward" spore protection mechanisms established during sporulation, such as Ca-DPA-complex, dehydration, A-DNA conformation, spore coat layers, spore core minerals, DNA binding by SASPs and the synthesis of the

DNA repair systems SP lyase and NHEJ, interact with “backward” spore repair mechanisms activated or induced during germination and outgrowth, such as the reactivation of ATP- and macromolecules-biosynthesis, (universal pathways or spore specific), active DNA repair of accumulated damage and *de novo* nucleotide/nucleic acid synthesis. This concerted interaction of “forward” spore protection and “backward” spore repair may constitute the major strategy of spores to maintain their high resistance towards a variety of hazardous stresses when attacked in the spore state. The identification of transcriptional and biochemical changes, elaborated in this work, has contributed substantial facets to this understanding.

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7. Internet (web links)

DSMZ, German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany
http://www.dsmz.de

National Institute for Radiological Sciences (NIRS), Chiba, Japan
http://www.nirs.go.jp/news/event/2006/04_03/biology.shtml

Genome News Network
http://www.genomenewsnetwork.org/resources/sequenced_genomes/genome_guide_index.shtml)

Genomes OnLine Database v 2.0
http://www.genomesonline.org

SubtiList World-Wide Web Server
http://genolist.pasteur.fr/SubtiList

Agilent Technology website
http://chem.agilent.com

ScanAlyze software
http://rana.stanfort.edu/software

ImaGene software - BioDiscovery
http://www.biodiscovery.com/index/imagene

Significance Analysis of Microarrays software
http://db.systemsbiology.net/software/VERAandSAM

National Center for Biotechnology Information
http://www.ncbi.nlm.nih.gov

DBTBS database
http:dbtbs.hgc.jp

UniProt Knowledgebase - SWISS-PROT
http://www.expasy.org/sprot

Protein Information Resource - PIR
http://pir.georgetown.edu

NRL3D Sequence-Structure Database / Protein Database - NRL3D
htttp://www.infobiogen.fr/db/NRL3D

Prokaryotic database of gene regulation and systems biology - PRODORIC
http://prodoric.tu-bs.de

Gene Ontology database - AmiGO
http://amigo.geneontology.org/cgi-bin/amigo/go.cgi

Kyoto Encyclopedia of Genes and Genomes - KEGG
http://www.genome.jp/kegg

JProGo website
http://www.jprogo.de

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Curriculum Vitae

Persönliche Daten

Ralf Möller

Geburtsdatum:	18. Oktober 1977
Geburtsort:	Suhl, Deutschland
Familienstand:	verheiratet
Kinder:	zwei

Ausbildung

1984 - 1989	5. Oberschule Werner Seelenbinder Schule in Suhl, Deutschland
1989 - 1994	Regelschule Lautenbergschule in Suhl, Deutschland
1994 - 1997	Gewerblich-Kaufmännisches Berufsbildungszentrum in Suhl-Zella/Mehlis und Suhl-Goldlauter, Deutschland

Studium

1997 - 1998	Biologie an der Universität Osnabrück, Deutschland
1998 - 2002	Biologie (mit Schwerpunkt Mikrobiologie, Biochemie, Biotechnologie und Ökologische Chemie) an der Technischen Universität zu Braunschweig, Deutschland
2002 - 2003	Diplomarbeit „Untersuchungen zur Wirksamkeit von endogenen und exogenen Schutzpigmenten auf die UV-Empfindlichkeit von Bakteriensporen“
2003 -	Promotion am Deutschen Zentrum für Luft- und Raumfahrt (DLR), Abteilung Strahlenbiologie in Köln, Deutschland mit dem Thema „Characterization of different types of radiation- and pressure-induced DNA damage in *Bacillus subtilis* spores and their global transcriptional response during spore germination“ im Rahmen einer Kooperation mit der Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Deutschland

Forschungsaufenthalte

2003 -	Max-Planck-Institut für Infektionsbiologie, Microarray Core Facility, Berlin, Deutschland
2003	Ernst-Moritz-Arndt-Universität Greifswald, Institut für Mikrobiologie, Abteilung für Mikrobielle Physiologie und Molekularbiologie, Greifswald, Deutschland
2004	University of Florida, Gainesville, Florida, USA and Department of Microbiology and Cell Science, Kennedy Space Center NASA, Cape Canaveral, Florida, USA
2005 -	International Space Radiation Laboratory, National Institute of Radiological Sciences, Chiba, Japan

Forschungsprojekte

2002 -	EVT-Experiments (Experiment Verification Tests) für DLR-ESA Projekt "EXPOSE" ("SPORES", ROSE experiment)
2003 - 2005	DFG Forschungsprojekt (HO 1508/3-1) "Meteorite ejection and microbial life (1)"
2005 - 2007	DFG Forschungsprojekt (RA 1049/1-2) "Meteorite ejection and microbial life (2)"
2005 -	HIMAC Projekt 17B463 "Gene activation of heavy ion treated *Bacillus subtilis* 168 (DSM 402) endospores during germination involved DNA-repair"
2005 -	DLR Projekt "HUMILAB - Behavior of terrestrial organisms under simulated Martian conditions"
2005 -	ESA/DLR Projekt "ADAPT"
2005 -	ESA/DLR Projekt "PROTECT"

Tagungsorganisation

2006	DFG Astro-/Exobiology Workshop "Viability in Space" DLR, Köln, Deutschland (Main Scientific Organizer)

Reviewer - Aktivität

2005 -	Current Microbiology
2006 -	Archives of Microbiology
2007 -	Canadian Journal of Microbiology