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# Type IV pili dependent DNA repair in Sulfolobales

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*Document Version*
Publisher's PDF, also known as Version of record

*Publication date:*
2013

Link to publication in University of Groningen/UMCG research database

*Citation for published version (APA):*
Ajon, M. (2013). *Type IV pili dependent DNA repair in Sulfolobales*. s.n.

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# Type IV pili dependent DNA repair in Sulfolobales

Małgorzata Ajon

Cover design: Katarzyna Warpas (www.kasiawarpas.blogspot.com)

Cover image: Jagoda Ajon

Layout and printing: Off Page, Amsterdam (www.offpage.nl)

ISBN: 978-94-6182-342-7

ISBN: 978-94-6182-344-1 (electronic version)

The work described in this book was carried out in the Department of Molecular Microbiology of the Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, the Netherlands and was financially supported by ALW grant from the Dutch Science Organization (NWO).

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# Type IV pili dependent DNA repair in Sulfolobales

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 1 november 2013
om 16.15 uur

door

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geboren op 22 september 1982
te Świdnica, Polen

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# 1

## HOW HYPERTHERMOPHILES ADAPT TO CHANGE THEIR LIVES: DNA EXCHANGE IN EXTREME CONDITIONS

Marleen van Wolferen, Małgorzata Ajon, Arnold J. M. Driessen and Sonja-Verena Albers

*Extremophiles (2013) 17(4):545-63*

### ABSTRACT

Transfer of DNA has been shown to be involved in genome evolution. In particular with respect to the adaptation of bacterial species to high temperatures, DNA transfer between the domains of bacteria and archaea seems to have played a major role. In addition, DNA exchange between similar species likely plays a role in repair of DNA via homologous recombination, a process that is crucial under DNA damaging conditions such as high temperatures. Several mechanisms for the transfer of DNA have been described in prokaryotes, emphasizing its general importance. However, until recently, not much was known about this process in prokaryotes growing in highly thermophilic environments. This review describes the different mechanisms of DNA transfer in hyperthermophiles, and how this may contribute to the survival and adaptation of hyperthermophilic archaea and bacteria to extreme environments.

# INTRODUCTION

Hyperthermophilic (superheat-loving) organisms are extremophiles and thrive at temperatures around 80°C or higher. The first hyperthermophile discovered in Yellowstone National Park by Thomas Brock was *Sulfolobus acidocaldarius* (Brock *et al.* 1972). Since then, over 90 hyperthermophilic species have been discovered (Stetter 2006a, b, 2013), most of them belonging to the domain of archaea, but some hyperthermophilic bacteria have also been characterized. Throughout evolution, hyperthermophilic organisms were able to adapt to changing environments such as up-shifts in temperature. Horizontal gene transfer (HGT) probably played an important role in the adaptation to more extreme environments. Except from a role in evolution, exchange of DNA might have also functioned in other mechanisms like DNA repair. DNA exchange is a widespread phenomenon that occurs in all domains of life. This emphasizes its significance for life on earth.

Three main mechanisms for transferring DNA have been described for archaea and bacteria: natural transformation, conjugation and transduction. Natural transformation is the uptake of DNA from the external environment, mostly emerging from lysed cells. Incoming DNA can be degraded and/or can be incorporated into the chromosomal DNA. The recipient cell is usually in charge of what DNA is and what DNA is not taken up (Chen *et al.* 2005; Lorenz and Wackernagel 1994; Thomas and Nielsen 2005). Conjugation on the other hand is a more invasive mechanism in which the donor has control over the transferred DNA. It requires direct contact between two cells that are not necessarily closely related. Mostly, small plasmids are transferred. Conjugation is suggested to be the main mechanism responsible for HGT (Halary *et al.* 2010; Norman *et al.* 2009; Wozniak and Waldor 2010). Transduction involves viruses that function as vehicles enabling DNA exchange between closely related species (Lang *et al.* 2012; Thomas and Nielsen 2005). Other mechanisms not belonging to these three include: the transfer of DNA via gene transfer agents (GTAs) which are virus-like elements encoded by the host genome (Lang *et al.* 2012), membrane vesicles (Gaudin *et al.* 2012) and nanotubes which are cellular protrusions that can bridge neighboring cells (Dubey and Ben-Yehuda 2011). Finally, DNA exchange has been shown to occur between hyperthermophilic *Sulfolobus* species (Grogan 1996). This DNA exchange is mediated by UV-inducible pili from *Sulfolobus* (Ups) (Fröls *et al.* 2008). The different DNA transfer methods among hyperthermophiles are listed in Table 1. This review will describe the fate and function of the DNA transferred between hyperthermophilic prokaryotes; furthermore, it will explain in what way the above-mentioned mechanisms contributed to DNA transfer between prokaryotes in hot environments.

**Table 1.** DNA transfer methods among (hyper)thermophiles

| | Organism | Mechanism | Mode | References |
|---|---|---|---|---|
| **Bacteria** | *Thermus thermophilus* | Conjugation | Exchange of megaplasmid pTT27 | (Alvarez *et al*. 2011; Ramírez-Arcos *et al*. 1998) |
| | *Thermus* species | Natural competence | Unspecific DNA uptake | (Gounder *et al*. 2011; Koyama *et al*. 1986) |
| | *Thermus thermophilus* | Possible transduction | Possible DNA transfer via viruses | (Ackermann and Prangishvili 2012) |
| **Archaea** | *Sulfolobus solfataricus, Sulfolobus islandicus* | Conjugation | Exchange of different conjugative plasmids | (Alvarez-Martinez and Christie 2009; Greve *et al*. 2004; Schleper *et al*. 1995) |
| | *Aciduliprofundum boonei* | Possible conjugation | Possible transfer of ICE | (Guglielmini *et al*. 2011) |
| | *Thermococcus kodakarensis* | Natural competence | Uptake of linear and circular DNA | (Sato *et al*. 2003) |
| | *Pyrococcus furiosus* | Natural competence | Uptake of linear and circular DNA | (Waege *et al*. 2010) |
| | *Sulfolobus acidocaldarius* | Natural competence? | Uptake of short oligonucleotides | (Grogan and Stengel 2008) |
| | *Sulfolobus* species | Ups-system | Bidirectional exchange of chromosomal DNA | (Ajon *et al*. 2011) |
| | *Thermococcus nautalis, Thermococcus kodakarensis* | Vesicles | Transfer of plasmid pTN1 and pCL70 | (Gaudin *et al*. 2012; Soler *et al*. 2011) |
| | *Thermococcus kodakarensis* | Nanotubes and nanopods | Possible DNA transfer | (Marguet *et al*. 2013) |
| | *Acidianus, Aeropyrum, Pyrobaculum, Stygiolobus, Sulfolobus, Thermoproteus, Pyrococcus, Thermococcus* | Possible transduction | Possible DNA transfer via viruses | (Ackermann and Prangishvili 2012) |

## HORIZONTAL GENE TRANSFER AND EVOLUTION

Bacteria and archaea are not able to reproduce sexually; instead they use binary fission for reproduction where DNA is transferred vertically from the mother to the daughter cells. In theory, this does not lead to genetic diversity. Yet, gene-mutation, -loss and -duplication can occur, introducing genetic variation. Moreover, archaea and bacteria can obtain DNA from their environment or nearby organisms and incorporate it into their own genomic DNA (Dickerson 1980). Early studies showing the spread of antibiotic resistances (Datta and Kontomichalou 1965) already made scientists believe that transfer of DNA occurred among bacteria. However, despite these observations, the importance of HGT in genome evolution was underappreciated for decades (Cohan 1994a, b). Therefore, early models of evolution were only based on vertical transfer of DNA (Levin 1981). Much later studies involving comparative genomics showed the extent to which HGT actually played a role in genome evolution (Jain *et al.* 2003; Koonin and Wolf 2008; Ochman *et al.* 2000). Nowadays it has become clear that throughout evolution, archaea and bacteria exchanged genes which allowed them to adapt to the changes in the dynamic environments they live in (Guglielmini *et al.* 2013).

Horizontally transferred DNA can be incorporated into the chromosome using different methods (Thomas and Nielsen 2005; Zaneveld *et al.* 2008). Besides homologous recombination (HR) between similar DNA molecules, which is executed by DNA repair proteins (described in the following section), other mechanisms exist. Integration can for instance be performed by site-specific integrases such as those found in the *Sulfolobus* spindle-shaped virus 1 (SSV1) or conjugative *Sulfolobus* plasmid pNOB8 (described in the “Transduction and HGT among hyperthermophilic viruses” and “Conjugation” sections, respectively) (She *et al.* 2004, 2006). Moreover, transposition might facilitate HGT (Zaneveld *et al.* 2008), transposons or insertion sequences (ISs) carrying genes from a previous host might integrate into the genome of another organism that contains the same transposable elements. HR between the ISs or transposons then leads to incorporation of the DNA. An example of the latter mechanism is a 16-kb DNA fragment flanked by ISs that has been transferred between *Pyrococcus furiosus* and *Thermococcus litoralis* (Diruggiero *et al.* 2000).

Bioinformatic methods designed to detect HGT, identify unusual base composition and codon usage within genomes (Garcia-Vallvé *et al.* 2000; Lawrence and Ochman 1998; Nakamura *et al.* 2004), or perform phylogenetic analyses on individual genes (Beiko *et al.* 2005; Puigbò *et al.* 2010). Studies using these methods estimated that at least 20% of the bacterial genes and 40% of the archaeal genes were transferred during evolution (Popa and Dagan 2011; Thomas and Nielsen 2005).

HGT occurs across species boundaries, even between organisms from different domains of life. The latter contributed tremendously to the adaptation of bacteria to hot environments, which is illustrated by the fact that hyperthermophilic bacteria obtained more genes originating from archaea than generally observed with mesophilic bacteria. For instance, the hyperthermophilic *Aquifex aeolicus* and *Thermotoga maritima* acquired 16.2 and 24% of their genes, respectively, from archaea. In comparison, mesophilic *Bacillus subtilis*, *Sunechocystis* species, *Borrelia burgdorferi* and *Escherichia coli* obtained only 5% or less archaeal genes (Aravind *et al.* 1998; Nesbø *et al.* 2001). Though it must be noted that HGT might, next to the cause of adaptation, also be the consequence of being hyperthermophily as was hypothesized by Nesbø *et al.* (2001).

The importance of HGT for adaptation to changing environments is illustrated by a genome comparison between *Deinococcus radiodurans* and *Thermus thermophilus* (Omelchenko *et al.* 2005). The two related species diverged from a common, probably mesophilic ancestor (Weisburg *et al.* 1989), but have surprisingly different phenotypes that correspond to their distinct natural habitats: *D. radiodurans* is a mesophile that is extremely resistant to ionizing radiation (Anderson *et al.* 1956), whereas *T. thermophilus* is a thermophile that shows a rather average sensitivity to ionizing radiation (Oshima and Imahori 1974). It was shown that in both species, adaptation to the highly dissimilar environments was mediated by the loss, and more importantly gain of several genes during evolution. *D. radiodurans* obtained bacterial genes that enhanced its ability to survive different kinds of stresses. *T. thermophilus* on the other hand, acquired numerous archaeal genes that contributed to its thermophilic adaptation. Of these, about 50% are encoded on a megaplasmid which has an increased plasticity. This is illustrated by the fact that between related *T. thermophilus* HB8 and T. *thermophilus* HB27, gene content and order of the plasmid differ substantially. However, both plasmids are implicated in growth at higher temperatures. They both encode several proteins involved in the putative mobile thermophilic-specific DNA repair system. In addition, a gene encoding a reverse gyrase was found on the megaplasmid of *T. thermophilus* HB8. On the other hand, in the megaplasmid of *T. thermophilus* HB27, a pseudogene of the reverse gyrase was found suggesting that this gene was once acquired from a hyperthermophilic organism by a common ancestor of the two strains, but was decayed from *T. thermophilus* HB27 (Brüggemann and Chen 2006; Omelchenko *et al*. 2005).

The reverse gyrase is found in many thermophiles and in all hyperthermophiles and is probably the best-documented example of HGT being involved in adaptation to hot environments (Déclais *et al.* 2000; Forterre 2002). Phylogenetic analyses suggest that an early archaeal reverse gyrase gene was acquired by an

ancient bacterium. This was then followed by distribution of the gene within the *Thermotogales* and *Aquificales* via HGT (Brochier-Armanet and Forterre 2007; Gribaldo and Brochier-Armanet 2006). Its contribution to life at higher temperatures is illustrated by the fact that a reverse gyrase deletion strain of *Thermococcus kodakaraensis* KOD1 shows growth defects especially at higher temperatures (Atomi *et al.* 2004). It might therefore be possible that it is only essential for life above 90°C. Moreover, moderately thermophilic *Nautilia profundicola* Am-H lives in an environment near hydrothermal vents with fluctuating temperatures; the reverse gyrase of this strain showed a significant upregulation upon up-shifts in temperature (Campbell *et al.* 2009). The reverse gyrase was previously thought to increase the DNA stability at high temperature by introducing positive coils into the DNA (Kikuchi and Asai 1984). However, not all hyperthermophiles have positively coiled DNA. More recent studies therefore suggest that the reverse gyrase is rather involved in the protection of DNA against degradation which occurs at high temperatures (Kampmann and Stock 2004; Napoli *et al.* 2004). Although the exact function of the reverse gyrase in this process is not yet fully resolved, its acquisition was almost certainly an important step in the evolution of many thermophilic bacteria (Heine and Chandra 2009).

Adaptations to environmental changes can also be rather subtle. On Vulcano Island (Italy) for instance, there are several shallow vents which have dissimilar and fluctuating physiological conditions (Capasso *et al.* 1999; Sedwick and Stuben 1996). The genome sequences of several *Pyrococcus* isolates from these vents revealed extensive genome rearrangements in specific genomic "hot spots" containing mobile genetic elements. It is therefore thought that a number of HGT events took place, possibly contributing to the adaptation to the rapidly changing environments on Vulcano Island (White *et al.* 2008).

Rachel Whitaker *et al.* used *Sulfolobus islandicus* as a model organism to study evolutionary biology in hyperthermophilic archaea (Zhang *et al.* 2013). By comparing the genomes of 12 different *S. islandicus* strains from a single hot spring in Kamchatka (Russia), they investigated the rate and mode of recent evolutionary events. Two coexisting groups of *S. islandicus* species were found that seem to have exchanged DNA mainly within the groups (Cadillo-Quiroz *et al.* 2012). This is a clear indication of speciation (Dykhuizen and Green 1991). Given the fact that *Sulfolobus* species exchange DNA in species-specific aggregates (Ajon *et al.* 2011), the barrier between the two *S. islandicus* groups could be the inability to successfully create mating pairs (Cadillo-Quiroz *et al.* 2012). This species-specific recognition is probably driven by ups-pili and glycosylation patterns (van Wolferen and Albers, unpublished), as will be described later. Strains from the two different *S. islandicus* groups might therefore show variations in these two structures. Future studies

need to confirm whether or not strains from the two different groups are indeed unable to form mixed aggregates. Moreover, glycosylation patterns and ups-pili between strains from the two different groups need to be compared.

Taken together, genome evolution has been essential for the adaptation of hyperthermophiles to changing and extreme environments in which HGT seems to have been the main player. Without the ability to exchange DNA between species, hyperthermophilic bacteria probably would not exist.

## DNA TRANSFER FOR REPAIR

Next to the crucial role DNA transfer played in genome evolution, other destinations of transferred DNA have become evident. It has been suggested that prokaryotes use imported similar DNA to repair their own DNA with HR (Bernstein *et al.* 1981). HR is the genetic recombination between two similar or identical molecules of DNA, and is the only efficient mechanism for accurately repairing double stranded DNA breaks (DSBs). It is dependent on another copy of the damaged DNA. This other copy can be the second chromosome that is naturally present during the G2 phase. However, taking up other DNA evidently increases the chances of having a non-damaged homologous template for DNA repair (Bernstein *et al.* 2012). Bernstein *et al.* (2012) even propose that DNA repair is the primary function of DNA transfer. For recent reviews describing the DSB repair via HR in bacteria and archaea, see Ayora *et al.* (2011) and White (2011).

Many bacteria induce their competence genes upon DNA damage which underlines the role that transferred DNA might play in DNA repair. In *Streptococcus pneumoniae*, competence is induced upon treatment with DNA damaging agents mitomycin C and fluoroquinolone (Claverys *et al.* 2006). Competence of *Helicobacter pylori* is induced by ciprofloxacin which causes DSBs (Dorer *et al.* 2010). *B. subtilis* shows DNA uptake upon UV stress (Michod *et al.* 1988), and *Legionella pneumophila* strongly induces competence upon the addition of several different DNA damaging agents (Charpentier *et al.* 2011). The only competence system studied in detail in thermophilic prokaryotes is that of *Thermus* species; this system, however, was shown to be constitutively active (Hidaka *et al.* 1994). Hyperthermophilic *Sulfolobus* species on the other hand, exchange DNA upon DSB induction with UV-light or chemical compounds, like bleomycin (Fröls *et al.* 2008). The mechanism behind this exchange is still unclear, but possibly it is related to the competence system as will be described later. Charpentier *et al.* (2011) speculate that DNA transfer might have evolved as a DNA damage response in SOS-deficient bacteria. Among the above-mentioned bacteria, *S. pneumoniae*, *H. pylori* and *L. pneumophila* indeed do not show an SOS response. This hypothesis might also be true for *Sulfolobus* species, as archaea

in general have so far not been shown to induce SOS responses. It remains to be shown if other hyperthermophilic bacteria and archaea also display DNA damage-inducible DNA transfer mechanisms that may or may not be similar to that found in *Sulfolobus* species.

Further evidence supporting the idea that transferred DNA functions in DNA repair is the fact that DNA uptake is often strongly biased towards DNA from the same or closely related species (Seitz and Blokesch 2012). In the Pasteurellaceae and Neisseriae it was shown that this bias is provoked by recognizing so-called DUS sequences that are present multiple times in their own DNA (Danner *et al.* 1980; Elkins *et al.* 1991; Fitzmaurice *et al.* 1984; Mell *et al.* 2012; van Passel 2008). In *Vibrio cholerae* on the other hand, the competence system is strongly induced by the presence of the species-specific auto inducer CAI-1, which also leads to a higher uptake of DNA from the same species (Suckow *et al.* 2011). Other bacterial species make use of restriction modification systems that degrade foreign DNA (Murray 2002) and increase thereby the chances of incorporating self-DNA. Likewise, CRISPR-Cas systems can target foreign DNA, and thereby hamper HGT, as was shown for *Staphylococcus epidermidis* (Marraffini and Sontheimer 2008). A recent study even showed that the presence of CRISPR loci seems to be linked to the presence of an active competence system (Jorth and Whiteley 2012). Uptake of self-DNA can additionally be increased by the induction of cell death of neighboring cells from the same species (fratricide), as has been shown for *S. pneumoniae* (Guiral *et al.* 2005; Håvarstein *et al.* 2006) and probably also *H. pylori* (Dorer *et al.* 2010). Lysed cells provide free DNA that can be taken up for possible DNA repair. Lastly, in the archaeal *Sulfolobus* species, DNA exchange occurs within species-specific cell aggregates (Ajon *et al.* 2011). In that way, only species-specific DNA is exchanged.

A bias towards species-specific DNA also occurs at the level of HR. In general, this process evidently gets less efficient for more dissimilar sequences to integrate into the DNA (Majewski and Cohan 1998, 1999; Wolf *et al.* 2001; Zawadzki *et al.* 1995). Therefore, in all prokaryotic species there is a natural bias towards integration of DNA from the same or closely related species.

In (hyper)thermophilic organisms one could hypothesize that DNA repair mechanisms are of particular importance as the rates of spontaneous DNA mutations are elevated at high temperatures (Lindahl 1993). However, the genomic mutation rate in the hyperthermophilic archaeon *S. acidocaldarius* was shown to be equal to mesophilic organisms (Grogan *et al.* 2001). Given the fact that DNA stability is more important at higher temperatures, mutations probably have a more drastic effect in hot environments. In (hyper)thermophiles, mutation rates should therefore theoretically be even lower than in mesophiles to give the

same effect. By extrapolating the mutation rate of used markers to the whole genome, recent studies indeed showed that the rate of base substitutions in *S. acidocaldarius* and *T. thermophilus* is lower compared to mesophilic organisms (Drake 2009). Spontaneous mutations in the DNA of (hyper)thermophiles must consequently be repaired so rapidly that they cannot be measured. Thus, efficient DNA repair systems seem to be present; this may involve efficient HR mechanisms that use transferred DNA as a template. In general, the role of DNA transfer in DNA repair of prokaryotes might be far more important than previously thought.

## DNA AS A NUTRIENT SOURCE

An alternative fate for imported DNA might be the use of nucleotides as a nutrient source (Palchevskiy and Finkel 2009; Redfield 1993a). This idea is supported by the fact that *B. subtilis* and *Haemophilus influenza* induce competence upon nutrient starvation and not upon, for instance DNA damage (Redfield 1993a, b). Moreover, non-homologous or partially degraded DNA molecules might not be suitable for HR and therefore better used as nutrient source (MacFadyen *et al.* 2001; Redfield *et al.* 1997; Redfield 1988). Proof for this theory is amongst others the observation that *E. coli* is able to grow with DNA as sole carbon and energy source, which is dependent on the presence of homologues of competence genes in *H. influenzae* and *Neisseria gonorrhoeae* (Finkel and Kolter 2001; Palchevskiy and Finkel 2006).

In hyperthermophilic environments, unprotected DNA is degraded faster than in mesophilic surroundings (Lindahl 1993). One could therefore speculate that bad-quality DNA that is commonly present is taken up by competent hyperthermophiles and used as a nutrient source. However, no evidences in agreement with this hypothesis have been shown so far. Moreover, it seems unlikely that the main function of DNA uptake is to serve as a food source when far less energy consuming processes are capable of taking up nutrients just as well. Hence, it seems more plausible that only DNA that cannot be incorporated into the genome is used as a source of nutrients.

## CONJUGATION

Conjugation is the unidirectional transfer of DNA between cells by a process requiring cell–cell contact (de la Cruz *et al.* 2010; Gomis-Rüth and Coll 2006). This process has a host range larger than observed for transformation or transduction (Guglielmini *et al.* 2013) and is thought to be the main mechanism involved in HGT (Halary *et al.* 2010; Norman *et al.* 2009; Wozniak and Waldor 2010). During

conjugation, DNA transfer is carried out by the donor cell by means of a bridge-like connection between two cells; the recipient cell seems to have little control in this process (Pérez-Mendoza and de la Cruz 2009). Mostly plasmids and ICEs (integrating conjugative elements) are transferred. Conjugation has thereby contributed significantly to the rapid spread of antibiotic resistance, virulence, and social traits among prokaryotes (Gomis-Rüth and Coll 2006; Guglielmini *et al.* 2013; Schröder and Lanka 2005).

Conjugation systems form the largest subfamily of the Type IV secretion systems (T4SS); the latter also include DNA uptake and release systems and protein translocation systems (Schröder and Lanka 2005). The T4SS is a large protein complex spanning the complete bacterial cell envelope. Proteins essential for T4SSs are: a TraU/VirB4 ATPase energizing both the assembly of the system as well as the substrate transfer, and 12–20 mating-pair formation proteins (MPFs) promoting physical contact with the recipient cell (Schröder and Lanka 2005). Besides the general T4SS proteins, conjugation-specific proteins include a relaxase and a type IV coupling protein (T4CP) (de la Cruz *et al.* 2010). The relaxase initiates conjugative transfer by binding and nicking the DNA at the origin of transfer (*oriT*). The T4CP then couples the DNA to the channel-forming T4SS that subsequently transfers the nucleoprotein complex through the membrane of the donor cell and delivers it into the recipient cell (de la Cruz *et al.* 2010; Vogelmann *et al.* 2011). A gene cluster encoding a VirB4 ATPase, a T4CP and a relaxase is considered to encode a putative conjugative system; without the relaxase, the system is thought to be a protein-exporting T4SS (Guglielmini *et al.* 2011).

In all different conjugation systems, assembly of the pilus is essential to draw cells close to each other and allow exchange of macromolecules from cell to cell. The best-characterized T4SS surface structures are the conjugative pili from Gram-negative bacteria. There is a huge diversity in composition and structure of these filaments. Pili encoded by the F plasmid from *E. coli* are long (2–20 μm) and flexible, with a diameter of 8 nm, while P-pili from IncP plasmid RP4 are very short, rigid and have diameters from 8 to 12 nm (Alvarez-Martinez and Christie 2009; Lawley *et al.* 2003; Silverman 1997).

**Conjugation in hyperthermophilic Bacteria**

Thermophilic *Thermus* species are equipped with a highly efficient natural competence system (described in the following section). Recently, it was speculated that also conjugation occurs among *Thermus* species (César *et al.* 2011). Given the low solubility of oxygen in geothermal environments, certain *T. thermophilus* strains express a nitrate reductase, which makes anaerobic growth possible. This nitrate reductase is encoded in the *nir-nar-nor* cluster, present as a variable region on megaplasmid pTT27 (Alvarez *et al.* 2011; Bricio *et al.* 2011). It was shown that

*T. thermophilus* strains NAR1, HB27 and PRQ25 were able to exchange pTT27 among each other, thereby enabling anaerobic growth (Alvarez *et al.* 2011; Ramírez-Arcos *et al.* 1998). In addition, when a hygromycin resistance marker (*hyg*) was placed on pTT27, this gene could be transferred to non-competent cells with frequencies of up to $10^{-2}$ (César *et al.* 2011). These findings support the presence of an active conjugation system. However, no conjugation-like homologous have been found in the above-mentioned *T. thermophilus* strains. Therefore, another unrelated conjugation system must be present in these species (César *et al.* 2011). A recently sequenced megaplasmid pTHTHE1601 from *T. thermophilus* SG0.5JP17-16 revealed the presence of a putative VirB operon, possibly involved in conjugation. Proteins encoded by this operon include TrbC/VirB2 (Ctr1), VirB4 (Ctr3) and VirD4 (Ctr7) ATPases. Future experiments need to confirm the participation of this T4SS in conjugation (César *et al.* 2011).

**Conjugation in hyperthermophilic Archaea**

Based on bioinformatics methods (hidden Markov models) scoring for co-localization of genes encoding a virB4/traU ATPase, a T4CP, a relaxase and MPF-specific proteins, only two archaeal conjugative elements could be found: one ICE encoded in the chromosome of the thermoacidophilic *Aciduliprofundum boonei*, and one conjugative plasmid in *Haloarcula marismortui* (pNG500). However, many VirB4 homologues were found encoded on archaeal chromosomes or plasmids, often associated with T4CP-like proteins. Because conjugative plasmids were found in *Sulfolobus* species (described below) it might be possible that unknown relaxases exist in archaea (Guglielmini *et al.* 2011).

Around 3% of all isolated *Sulfolobus* strains contain self-transmissible conjugative plasmids (Prangishvili *et al.* 1998). The best-characterized plasmids are pNOB8 from Japanese *Sulfolobus* strain NOB8H2 (Schleper *et al.* 1995; She *et al.* 1998) and pING1 isolated from *S. islandicus* strain HEN2P2 (Prangishvili *et al.* 1998; Stedman *et al.* 2000). The first archaeal plasmid shown to be horizontally transferred is a relatively large plasmid (45 kb) pNOB8. It can propagate easily in liquid cultures of its original host in mating mixtures with *Sulfolobus solfataricus* and *S. islandicus* via conjugative-like transfer mechanism. pNOB8 has very high copy numbers of between 20 and 40 plasmids per chromosome. When pNOB8 is transformed to *Sulfolobus* cells; the cells become a donor and are able to transfer the plasmid to other cells. The same was observed for recipient cells containing the transmitted plasmid (Schleper *et al.* 1995). Interestingly, upon mixing a donor and recipient strain, cellular aggregates could be observed. Moreover, electron microscopy revealed intercellular cytoplasmic bridges that connect two or more cells (Figure 1A) (Schleper *et al.* 1995), which resemble those found in halophilic *Haloferax volcanii*. The cytoplasmic bridges of *H. volcanii* mediate the bidirectional

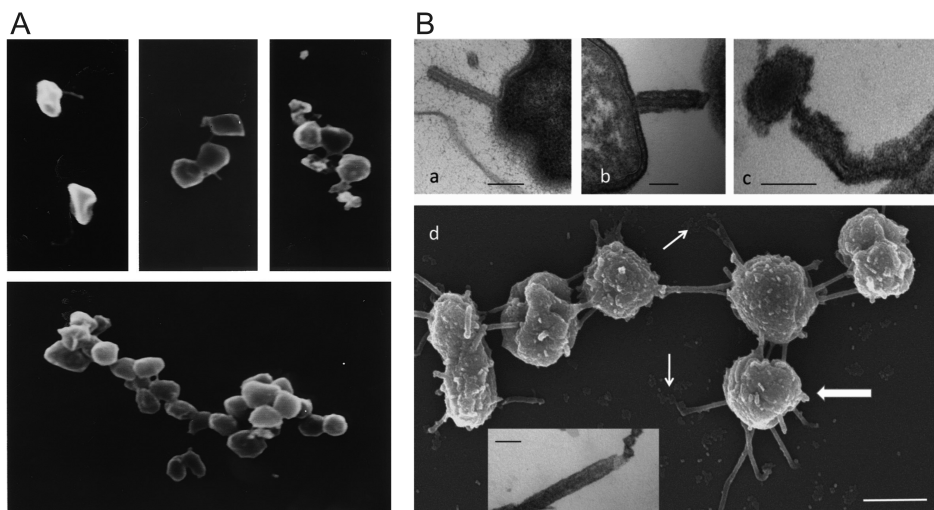


**Figure 1.** A: Scanning electron micrographs of donor strain *S. solfataricus* NOB8H2 (*upper left*) and 1:1 mating mixtures with *S. solfataricus* NOB8H23, showing cell aggregates and intercellular bridges. Reprinted from Schleper *et al.* (1995). B: Transmission electron micrographs from cross sections of nanotubes from *Thermococcus* sp, *scale bar* 200 nm (a) and 100 nm (b) (a and b). Nanotube associated with a MV, *scale bar* 100 nm (c). Scanning electron micrograph of a network of cells linked by nanotubes, *scale bar* 500 nm. *Inset*: partially disrupted nanotube showing an artificially twisted internal core extruding from an outer envelope; scale bar, 100 nm) (d). Reprinted from Marguet *et al.* (2013).

transfer of chromosomal DNA representing the first known mating system in archaea (Rosenshine *et al.* 1989). pNOB8 was also shown to be able to integrate site-specifically into the host genome within a tRNA gene (in the so-called *attP* site) using a self-encoded integrase (She *et al.* 2004).

Several other archaeal conjugation plasmids have been characterized during the last two decades. All were found in *Sulfolobus* species and they are grouped into pKEF or pARN plasmids (Alvarez-Martinez and Christie 2009; Greve *et al.* 2004). In all analyzed conjugative plasmids only a few genes encode proteins homologous to bacterial conjugation proteins, including TraG/VirD4, TrbE/VirB4 and partitioning proteins ParA and ParB. Therefore, despite the fact that conjugational exchange of plasmids has been observed between *Sulfolobus* species, the exact mechanism is still unclear.

Importantly, *Sulfolobus* species were also shown to bidirectionally exchange chromosomal DNA, possibly via a conjugation-like mechanism (Aagaard *et al.* 1995; Ajon *et al.* 2011; Ghane and Grogan 1998; Schmidt *et al.* 1999). This mechanism is mediated by UV-inducible pili and will be discussed separately in one of the following sections.

Overall, conjugational exchange of plasmid DNA has been observed both in hyperthermophilic bacteria and archaea; however, only very few of the classical conjugation proteins are found in these species. It seems therefore that conjugation at high temperatures involves different specialized machineries that so far have not been identified and characterized.

## NATURAL TRANSFORMATION

Natural transformation refers to the uptake of exogenous DNA by naturally competent species (Dubnau 1999). It is a widespread phenomenon in bacteria that has been described for at least 70 bacterial species from all major taxonomic groups. In contrast to conjugation, competence does not require mobile genetic elements (Johnsborg *et al.* 2007). As previously discussed, imported DNA can be used for different cellular processes, such as DNA repair, nutrition and the introduction of genetic diversity (Seitz and Blokesch 2012). The process of natural transformation starts with the induction of competence, followed by the binding of environmental DNA, which is subsequently fragmented whereupon it enters the cells. If the entered DNA shows homology to certain genomic DNA regions it can be integrated into the chromosome (Averhoff 2009).

Despite the fact that competence systems are common in bacteria, initiation and regulation of the systems differ between species. Some organisms have highly regulated competence that is triggered by certain signals like pheromones, nutrient limitation or high cell density. Competence in Gram-positive *B. subtilis* and *S. pneumonia* has been studied in detail and seems to be a general response to stress (Johnsborg *et al.* 2007; Lorenz and Wackernagel 1994). In Gram-negative bacteria, regulation of competence is less well understood. However, it is known that *H. influenza* induces competence upon starvation (Herriott *et al.* 1970) and *Vibrio cholera* upon growth on chitin (Meibom *et al.* 2005). Moreover, as described previously, many bacteria show induction of competence upon DNA damage (Charpentier *et al.* 2011; Dorer *et al.* 2010; Michod *et al.* 1988), and thus competence may serve for DNA repair via HR. The only well-studied thermophilic bacteria harboring natural competence systems belong to the genus *Thermus*. These competence systems seem to be constitutively active (Hidaka *et al.* 1994), as was also shown for mesophilic *N. gonorrhoeae* (Dubnau 1999). Bacterial competence is in general subject to regulation by complex signal transduction pathways [for reviews see Claverys *et al.* (2006); Seitz and Blokesch (2012)].

Due to their vastly different cell envelopes, DNA uptake mechanisms in Gram-positive and -negative bacteria structural differ, in particular, the presence of a secretin ring is unique for the outer membrane of Gram-negative bacteria. However, most of the other proteins are shared (reviewed in Krüger and Stingl

2011) indicating a large similarity in DNA uptake mechanisms. Most bacterial competence systems are composed of a DNA-translocation complex that is coupled to a type IV pilus (T4P) or an evolutionary related type II secretion system (T2SS), proteins in both systems include a pre-pilin peptidase, a secretion ATPase, a polytopic transmembrane protein and pilin- or pseudopilin-subunits. The DNA uptake systems of *H. pylori* and *Campylobacter jejuni* form an exception as they are related to the dissimilar T4SSs (described in the "Conjugation" section) (Hofreuter *et al.* 2000; Stingl *et al.* 2009).

The exact role of the pili or pseudopili in competence is still not well understood, but their presence is essential for successful DNA uptake (reviewed in Krüger and Stingl 2011). It has been suggested that DNA is brought close to the cell surface by binding to the (pseudo)pili that subsequently retract. Until recently, this hypothesis was questioned as no binding of DNA to (pseudo)pili had ever been observed (Assalkhou *et al.* 2007; Provvedi and Dubnau 1999). However, a very recent paper described the binding of minor pilin ComP of competent *Neisseria meningitidis* to DUS sequences suggesting that pili are indeed bringing DNA to the cell surface (Cehovin *et al.* 2013). For detailed reviews about bacterial competence systems, see Averhoff and Friedrich (2003), Chen and Dubnau (2004), Claverys *et al.* (2009), Johnsborg *et al.* (2007) and Krüger and Stingl (2011).

**Naturally competent hyperthermophilic Bacteria**

Of the at least 70 naturally competent bacteria that are found so far (Johnsborg *et al.* 2007), six are (hyper)thermophilic. One of these is the thermophilic cyanobacterium *Thermosynecoccus elongates* BP-1 (Onai *et al.* 2004), all others belong to the *Thermus* genus, these are: *T. aquaticus*, *T. caldophilus*, *T. flavus*, *T. thermophilus* (Koyama *et al.* 1986) and *T. scotoductus* (Gounder *et al.* 2011). With transformation frequencies of up to $10^{-2}$, hyperthermophilic *T. thermophilus* was shown to have the most efficient DNA uptake system of all studied naturally competent bacteria (Koyama *et al.* 1986). This was confirmed by the fact that DNA uptake had a speed of around 40 kb/s per cell, which is extremely fast (Schwarzenlander and Averhoff 2006). In comparison, other bacteria like *B. subtilis* and *H. influenza* show rates of 4 and 16 kb/s per cell, respectively (Deich and Smith 1980; Dubnau 1991). *T. thermophilus* has for that reason become a model organism for studying natural transformation in bacteria.

Additional properties that make the *T. thermophilus* DNA uptake system of great interest is that it is constitutively active (Hidaka *et al.* 1994) and that it is equipped with broad substrate specificity. *T. thermophilus* HB27 does not show any bias towards certain types of DNA; genetic material from all domains of life can be taken up with equal efficiencies (Schwarzenlander *et al.* 2009; Schwarzenlander and Averhoff 2006). From this point of view, one could speculate that the *Thermus*

competence system is involved in HGT rather than in DNA repair. This hypothesis is strengthened by the fact that throughout evolution *Thermus* species have indeed undergone several HGT events; these events are suggested to be involved in the gain of thermophilicity (Averhoff and Müller 2010; Omelchenko *et al.* 2005). It is therefore hypothesized that *Thermus* species developed a highly efficient competence system to acquire all sorts of DNA, which allowed them to adapt through the incorporation of foreign, probably primarily archaeal, genes.

Through whole-genome comparisons and mutational analyses, 16 competence genes were identified in *T. thermophilus* (Friedrich *et al.* 2001, 2002, 2003). Genome comparisons further revealed that these genes are conserved among all sequenced *Thermus* species (César *et al.* 2011). The proteins encoded by those genes are divided into three groups: type IV pili-related proteins (PilA1–4, PilD, F, C, Q), DNA translocator proteins (ComEA, ComEC, DprA) and novel *Thermus*-specific proteins (ComZ, PilM, N, O, W) (Averhoff 2009) (Figure 2A). Among the type IV

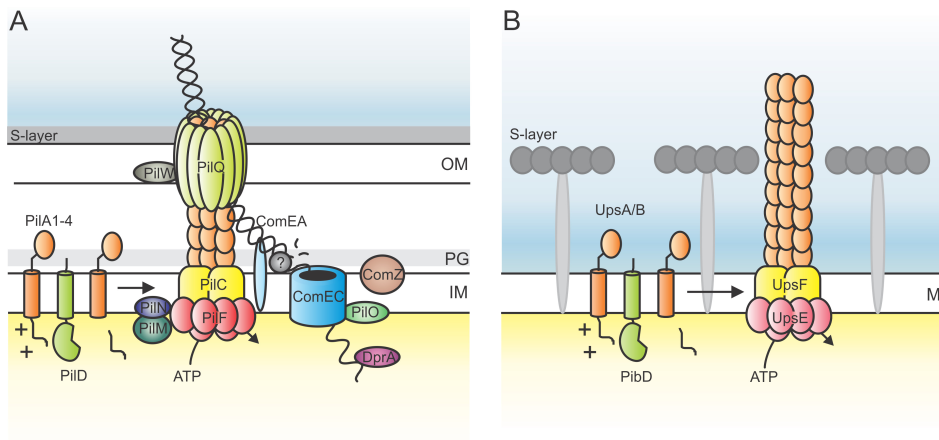


**Figure 2.** Proposed DNA transfer systems in (hyper)thermophilic *Thermus* and *Sulfolobus* species. A: The natural competence system of *T. thermophilus*: Pilin subunits PilA1-4 are processed by signal peptidase PilD and subsequently build into a pilus. PilC anchors the structure into the inner membrane. DNA enters the cell envelope via outer membrane protein PilQ. Upon pili retraction energized by the ATPase PilF, DNA enters the periplasmic space and it is handed over by ComEA to channel forming ComEC transporting the DNA further into the cytoplasm. Before entering the cell, one strand of the DNA is degraded by a yet unknown nuclease while the other strand of incoming DNA is guided by DprA to RecA. Accessory proteins PilM, PilN, PilO, PilW and ComZ, are either involved in the assembly of the transporter and/or assist transport of DNA across the inner membrane. B: The UV-inducible pili system in of Sulfolobales (ups-system): Pilin subunits UpsA and UpsB are processed by signal peptidase PibD and subsequently build into a pilus, energized by ATPase UpsE. UpsF anchors the structure into the membrane. *OM*: outer membrane, *PG*: peptidoglycan, *IM*: inner membrane, *M*: membrane.

pili-related proteins there is a conserved pre-pilin signal peptidase PilD which is, as was shown in many other bacteria and archaea, responsible for the maturation of the pilin subunits (Nunn and Lory 1991). Four predicted pilin subunits are present: PilA1, PilA2, PilA3 and PilA4, which are essential for pili formation (Friedrich *et al.* 2002). So far, no ATPase energizing the assembly of the type IV pili has been identified in *T. thermophilus.* However, a deletion mutant of a second traffic-ATPase, PilF, resulted in piliated but non-competent cells (Friedrich *et al.* 2001), PilF is therefore thought to be a retraction ATPase functioning similar to PilT in *N. gonorrhoeae* (Maier *et al.* 2002; Wolfgang *et al.* 1998). Other type IV pili-related proteins are: the inner membrane protein PilC, anchoring the pili into the inner membrane, and the outer membrane protein PilQ whose structure was recently elucidated (Burkhardt *et al.* 2011). The latter is similar to secretins in other Gram-negative bacteria and was shown to bind DNA. PilQ is thought to form a membrane channel that guides the DNA through the outer membrane to the DNA translocator which is present in the inner membrane (Burkhardt *et al.* 2012; Rumszauer *et al.* 2006; Schwarzenlander *et al.* 2009).

The three DNA translocator proteins found in *T. thermophilus* are all homologous to conserved competence proteins of other bacteria: ComEA is a protein anchored to the inner membrane that binds DNA and subsequently guides it to ComEC. ComEC forms a channel in the inner membrane and transports the DNA into the cytosol. During this process, incoming DNA is probably linearized by an unknown nuclease that degrades one of the two strands. Single stranded DNA is subsequently thought to be handed over to RecA by DprA as shown for *S. pneumonia* (Mortier-Barrière *et al.* 2007). As RecA is essential for transformation (Martin *et al.* 1995), transferred DNA might then be integrated into the genomic DNA via a RecA-dependent process.

Not much is known about the *Thermus*-specific competence proteins; PilM, N, O and ComZ are all present in the cytoplasmic membrane (Friedrich *et al.* 2003; Rumszauer *et al.* 2006) and are suggested to be involved in the assembly of the DNA-translocation system (Averhoff and Müller 2010). PilW is thought to be responsible for proper localization of PilQ in the outer membrane (Rumszauer *et al.* 2006). As *T. thermophilus* lives at extremely high temperatures, the unique competence proteins might be an evolutionary adaptation to these special environmental conditions (Averhoff and Müller 2010). For detailed reviews about the competence system in *T. thermophilus* see, Averhoff (2009), Averhoff and Müller (2010) and César *et al.* (2011).

**Naturally competent hyperthermophilic Archaea**

Only four archaeal species have so far been found to be naturally competent (Bertani and Baresi 1987; Sato *et al.* 2003; Waege *et al.* 2010; Worrell *et al.* 1988),

including two hyperthermophilic organisms. The first naturally transformable hyperthermophilic archaeon was *Thermococcus kodakarensis* (Sato *et al.* 2003), a sulphur reducing euryarchaeon that lives in marine hydrothermal vents and has an optimal growth temperature of 85°C. It can be transformed naturally with linear and circular DNA, which is subsequently integrated into the genomic DNA. The observed transformation frequencies of $10^{-7}$ are rather low (Sato *et al.* 2003, 2005). Another naturally competent member of the Thermococcales is *P. furiosus* which grows at temperatures around 100°C (Waege *et al.* 2010). Also this species can be transformed with linear and circular DNA and shows transformation frequencies of up to $10^{-3}$ approaching those of highly competent bacteria (Lipscomb *et al.* 2011). Among the crenarchaeota, so far no naturally competent organisms have been found. Nevertheless, Grogan and Stengel (2008) were able to show that the hyperthermophilic *S. acidocaldarius* is able to constitutively take up short single stranded oligonucleotides. Though, as earlier experiments revealed that no DNA uptake could be observed when cells were mixed with purified marker DNA, culture supernatant or lysed cells (Grogan 1996), one could question if we can speak here about true natural competence for *S. acidocaldarius*.

The mechanisms of natural transformation in (hyperthermophilic) archaea have not been studied, and the systems involved have not been identified as homologs of bacterial competence systems have so far not been found (Averhoff 2009; Claverys *et al.* 2009). However, the complete DNA sequence of hyperthermophilic crenarchaeon *Thermoproteus tenax*, revealed the presence of a few homologs of bacterial genes involved in competence (*dprA* and *comF*). Since these have not been detected in any other archaea, they were probably acquired from bacteria via HGT (Siebers *et al.* 2011). However, the existence of a functional bacterial-like competence system in *T. tenax* needs to be proven. It is likely that naturally transformable archaea that did not acquire competence genes from bacteria have other unrelated DNA uptake mechanisms, which will be a challenge for future studies.

## TRANSDUCTION AND HGT AMONG HYPERTHERMOPHILIC VIRUSES

Transduction refers to the transfer of DNA from one cell to another by means of viruses and was first described for *Salmonella typhimurium* (Zinder and Lederberg 1952). In this process, viruses accidentally package host DNA along with their own DNA and subsequently infect other prokaryotes. In that way, surviving host cells might acquire genetic material from previous hosts (Canchaya *et al.* 2003). Transduction therefore probably contributed significantly to HGT. Whole-genome

analyses indeed suggest that phages played an important role in the acquisition of new genes thereby promoted genetic diversity (Bordenstein and Reznikoff 2005; Brüssow *et al.* 2004; Ochman *et al.* 2000; Pallen and Wren 2007). Moreover, when two different viruses infect the same host simultaneously, HGT might not only occur between previous and current hosts, but also among the viruses (Krupovic *et al.* 2011), leading to viral genetic diversity.

Similar to viruses, gene transfer agents (GTAs), which are virus-like elements encoded by the host-genome, contribute to HGT. It has been demonstrated that GTAs can horizontally transfer a kanamycin resistance gene very efficiently between bacterial species (McDaniel *et al.* 2010), which implies a general importance of GTAs in HGT. However, so far no GTAs have been found in hyperthermophilic organisms.

Since 1959, about 6,300 prokaryotic viruses have been described morphologically (Ackermann 1996, 2001, 2007; Ackermann and Prangishvili 2012; Eisenstark 1967). Most of these are bacterial viruses (98.5%), however, over the past few years increasing numbers of archaeal viruses have been described, especially those infecting hyperthermophilic crenarchaea. All studied hyperthermophilic viruses were shown to contain DNA and not RNA, but a recent study revealed the presence of viral RNA in acidic hot springs in Yellowstone National Park, USA, probably originating from viruses infecting hyperthermophilic archaea. These unidentified RNA viruses might form a novel group of phages (Bolduc *et al.* 2012). For a detailed review about bacterial and archaeal viruses, see Krupovic *et al.* (2011).

**Viruses infecting hyperthermophilic Bacteria**

Within the (hyper)thermophilic bacteria, so far only a relatively small number of phages has been found. In total 27 hyperthermophilic bacteriophages have been morphologically described, all infecting *Thermus* species (Ackermann and Prangishvili 2012). These include*: Inoviridae*, *Myoviridae*, *Siphoviridae*, and *Tectiviridae.* The genomes of myovirus φYS40 (Naryshkina *et al.* 2006; Sakaki and Oshima 1975), siphoviruses P23-45, P74-26 (Minakhin *et al.* 2008; Yu *et al.* 2006), IN93 and icosahedral virus P23-77 (Jaatinen *et al.* 2008; Jalasvuori *et al.* 2009) were sequenced which contributed greatly to the understanding of regulation of transcription and translation as well as the evolution of *Thermus* phages. The sequence of P23-77 showed an evolutionary link to another *Thermus* phage, IN93. Interestingly, it also revealed evolutionary relationships to *haloarchaeal* plasmid pHH205, an integrated *Haloarcula* provirus, and the *Haloarcula* virus SH1. These similarities include homologies between two major capsid proteins and a putative packaging ATPase (Jalasvuori *et al.* 2009). A possible explanation for their relatedness could be that the haloarchaeal and *Thermus* viruses evolutionarily diverged from each other along with their hosts. Subsequently, the viruses may

have evolved into plasmids or proviruses. Another hypothesis is that viruses were able to cross the domain barrier and could thereby mediate HGT between the two domains (Jalasvuori *et al.* 2009). In the course of this process, other host genes might also have been transferred.

The DNA sequence from another bacteriophage φIN93 revealed the presence of an IS that was shown to originate from its host *T. thermophilus* (Matsushita and Yanase 2009). Upon a future infection, the virus might again transfer this IS to another organism, leading to HGT.

**Viruses infecting hyperthermophilic Archaea**

Among the studied hyperthermophilic viruses, most were found to infect members of the crenarchaeota, including: *Acidianus*, *Aeropyrum*, *Pyrobaculum*, *Stygiolobus*, *Sulfolobus* and *Thermoproteus* species. These viruses were shown to be highly diverse both on morphological and genomic level and have been classified into eight viral families: *Ampullaviridae*, *Bacaudaviridae*, *Clavaviridae*, *Fuselloviridae*, *Globuloviridae*, *Guttaviridae*, *Lipothrixviridae* and *Rudiviridae* (Ackermann and Prangishvili 2012).

The spindle-shaped fuselloviruses that infect *Sulfolobus* and *Acidianus* species are among the best-studied crenarchaeal viruses (Lipps 2006). The genome of *Sulfolobus* spindle-shaped virus 1 (SSV1), isolated from *Sulfolobus shibatae* was the very first archaeal viral genome being sequenced (Palm *et al.* 1991). Since then, 13 more fusellovirus genome sequences have been published (Held and Whitaker 2009; Redder *et al.* 2009). As was shown for conjugative plasmid pNOB8, SSVs can stably integrate in a *Sulfolobus* chromosome via integration within a tRNA gene (in the so-called *attP* site), thereby forming a provirus (Held and Whitaker 2009; Muskhelishvili *et al.* 1993). This site-specific integration is thought to be an important mechanism for HGT and genome evolution. The SSV1-type integrase that is essential for this integration, is next to the pNOB8-type integrase (described in the "Conjugation" section) of particular interest in studies on the integration of crenarchaeal mobile genetic elements (She *et al.* 2006; She *et al.* 2004). SSVs might in addition be involved in the HGT of plasmids; upon an SSV-infection, non-conjugative pRN-like plasmids could be encapsulated into virus-like particles that are released from the cell. Subsequently, they can spread with the help of the viruses and integrate into a following host genome using a self-encoded SSV1-type integrase (Arnold *et al.* 1999; Wang *et al.* 2007). Although they cannot infect *S. acidocaldarius*, SSVs are not host species dependent, it was shown that they can infect different Sulfolobales (Ceballos *et al.* 2012). Consequently, virus-mediated HGT might have occurred between the different hosts of SSVs (Ceballos *et al.* 2012).

Next to the *Fuselloviridae*, members from the *Rudi-* and *Lipothrixviridae* also infect *Sulfolobus* and *Acidianus*species; these are helical viruses with a linear dsDNA

genome (Prangishvili *et al.* 2006a). Comparative genomics revealed that members from the two virus families exchanged genes horizontally. Interestingly, it was shown that both viruses also obtained genes from their hosts (Peng *et al.* 2001). The latter include genes encoding a dUTPase: a thymidylate synthase (ThyX) and a Holliday junction resolvase (Peng *et al.* 2001; Prangishvili *et al.* 2006b). This acquisition of host genes could have possibly led to HGT between different hosts. Comparable to the fusseloviruses, a lipothrixvirus also shows a relationship with an integrative plasmid. *Acidianus* filamentous virus 1 (AFV1) mediates the horizontal spread of pAH1 between different hosts. The mechanism involved is so far not known (Basta *et al.* 2009).

Other crenarchaeal viruses involved in HGT are the *Sulfolobus* turreted icosahedral viruses (STIVs). A sequence comparison between STIV and related STIV2, revealed a similar genome organization as well as the loss and gain of several genes (Happonen *et al.* 2010). STIV2 shares genes with other archaeal viruses, including the *Fuselloviridae*, and it also contains a conserved crenarchaeal gene encoding a DNA-binding protein. Moreover, *Thermococcus kodakarensis* virus 4 (TKV4) and *Methanococcus voltae* virus (MVV) were shown to encode capsid proteins and genome packaging ATPases that were similar to those from STIV-like viruses (Krupovic and Bamford 2008). It is therefore clear that HGT has occurred between these archaeal viruses (Koonin 2009; Koonin and Wolf 2008). This transfer of DNA might have been mediated by viruses structurally related to STIV able to infect both eury- and crenarchaeota (Krupovic *et al.* 2011).

Only two hyperthermophilic euryarchaeal viruses have been described, both infecting members from the order of thermococcales: *Pyrococcus abyssi* virus 1 (PAV1) (Geslin *et al.* 2003, 2007) and *Thermococcus prieurii*virus 1 (TPV1) (Gorlas *et al.* 2012). They are morphologically similar to the *Fuselloviridae* and infect cells without causing lysis. Interestingly, the PAV1 genome shows homology to several different plasmids and integrating elements from other archaeal species; these genes are all encoded on approximately the same half of the viral genome. Genes encoding capsid proteins are located on the other half of the genome. This genetic organization could be due to a fusion of a plasmid and a virus, or otherwise, PAV1 might have given rise to plasmids and integrating elements. As was described for SSV1 and AFV1 viruses, PAV1-like viruses might have been involved in the horizontal transfer of plasmids (Krupovic *et al.* 2010).

In general, it seems clear that virus infections contributed significantly to HGT between viruses as well as their hosts. In the course of evolution this has led to a highly diverse virus population as well as dynamic prokaryotic genomes. Future studies might give more detailed insights in the impact that viruses have on the evolution of their hosts especially in hyperthermophilic environments.

## MEMBRANE VESICLES, NANOPODS AND NANOTUBES

The release of membrane vesicles (MVs) is an important physiological process for organisms from all domains of life. MVs mediate the intercellular transfer of several different biological compounds including DNA. They might therefore have contributed to HGT. Both bacterial and archaeal MVs are produced from the cell surface (Deatherage and Cookson 2012). Not much is known about how this is achieved in Gram-positive bacteria, but in Gram-negative bacteria the release of vesicles is thought to be promoted by temporal disruptions of the interaction between the outer membrane and the peptidoglycan (Deatherage and Cookson 2012). In archaea on the other hand, in Sulfolobales the release of MVs appears to occur by membrane scission events that are facilitated by ESCRT-III proteins (Ellen *et al.* 2009). In other archaeal species that do not contain ESCRT-III homologues, such as *Thermococcus*, the mode of vesicle formation is unknown. For recent reviews about MV formation in bacteria and archaea, see Deatherage and Cookson (2012), Kulp and Kuehn (2010) and Mashburn-Warren and Whiteley (2006).

Several bacteria were shown to produce DNA-containing vesicles (Dorward *et al.* 1989; Kahn *et al.* 1982; Kolling and Matthews 1999; Pérez-Cruz *et al.* 2013; Renelli *et al.* 2004; Rumbo *et al.* 2011; Yaron *et al.* 2000). However, no MV release has been observed for hyperthermophilic bacteria. Hyperthermophilic archaea on the other hand do generate MVs. *Sulfolobus* species were shown to produce MVs harboring toxins killing other *Sulfolobus* strains (sulfolobicins) (Ellen *et al.* 2009; Prangishvili *et al.* 2000). Moreover, *Ignicoccus* species produce many periplasmic MVs (Rachel *et al.* 2002). Euryarchaeota from the order of *Thermococcales* commonly release MVs that were previously thought to be viruses. Some of these MVs were shown to be associated with DNA that is highly resistant to DNAse treatment and high temperatures (Soler *et al.* 2008). MVs from *Thermococcus nautilus* contain the endogenous plasmid pTN1 (Soler *et al.* 2011). If a derivative from this plasmid, shuttle vector pLC70 (Santangelo *et al.* 2008), is transformed to *T. kodakarensis*, the cells start to release MVs containing the same plasmid. These MVs can be subsequently used again to transfer pLC70 into plasmid-free cells. As the transfer of DNA was insensitive to DNAse treatment, DNA must have been present inside the MVs. MVs might therefore function in both the protection as well as the transfer of DNA (Gaudin *et al.* 2012). Comparative genomics showed that HGT has taken place between *Thermococcus* and *Thermotoga*. Moreover, it is thought that certain plasmids have been horizontally transferred between the Thermococcales and Methanococcales (Krupovic *et al.* 2013). Future studies need to determine whether or not MVs can also be transferred efficiently between different species. This would imply an important role of MVs in the above-described HGT events (Marguet *et al.* 2013).

An interesting structure, first described for the Gram-negative soil bacterium *Delftia* sp. Cs1, is the so-called nanopod (Shetty *et al.* 2011). Nanopods are prokaryotic organelles, used for projecting MVs several micrometers away from the cell. Very recently, both *T. gammatolerans* and *T. kodakarensis* were shown to have tubular structures with a row of internal vesicles resembling bacterial nanopods (Marguet *et al.* 2013). Similar to the nanopods from *Delftia* sp. Cs1, the nanopods from *Thermococcus* species might also project MVs and could thereby increase the distance and perhaps also efficiency of MV transfer between cells (Marguet *et al.* 2013). Next to nanopods, also nanotubes can be found in Thermococcales (Marguet *et al.* 2013). Nanotubes are intra- or inter-species tubular cytoplasmic bridges between neighboring cells and were first described for *B. subtilis, S. aureus* and *E. coli*. As the structures are between 30 and 100 nm wide, all sorts of molecules including DNA can be transferred between connected cells (Dubey and Ben-Yehuda 2011). Very recently, nanotubes with a diameter of 60–80 nm have been observed in the hyperthermophilic *Thermococcus* sp. 5-4 (Marguet *et al.* 2013) (Figure 1B). Given their similar appearance, these archaeal structures might also be involved in exchange of biomolecules, as is observed for bacterial nanotubes. In that way they might facilitate DNA transfer between cells from similar or different species. As nanotubes now have been observed in a range of bacteria and archaea, they appear to be more widespread than previously thought. Other tubular structures observed in hyperthermophilic archaea are the cannulae found between *Pyrodictium* cells (König *et al.* 1988). Cryo-electron tomography, however, revealed that the cannulae enter the periplasmic space, but not the cytoplasm (Nickell *et al.* 2003). It is therefore unlikely that they transfer DNA.

## UV-INDUCIBLE PILI OF SULFOLOBALES (UPS)

A further DNA transfer mechanism that involves type IV pili is a UV-induced system in hyperthermophilic Sulfolobales (Schmidt *et al.* 1999). The type IV pili that are involved in this DNA exchange are the so-called ups-pili (UV-inducible pili of Sulfolobales) (Fröls *et al.* 2008). These pili were shown to mediate UV-induced cellular aggregation during which chromosomal marker exchange could be observed. With marker recombination frequencies of up to $10^{-2}$ the efficiency of DNA transfer is very high (Ajon *et al.* 2011). Next to UV stress, the DNA damaging agent bleomycin could also induce cellular aggregation. Thus, the trigger for pili formation and subsequent cellular aggregation is therefore probably the formation of DNA DSBs (Fröls *et al.* 2008). Since *ups*-knockout strains showed decreased survival rates upon UV treatment (Ajon *et al.* 2011), the ups-pili are proposed to be part of a unique HR-based "community" DNA repair mechanism (Fröls *et al.* 2009). Correspondingly, the formation of cellular aggregates only takes place between

cells from the same *Sulfolobus* species ensuring species-specific DNA exchange essential for DNA repair (Ajon *et al.* 2011). Recent data suggest that the intraspecies recognition of *Sulfolobus* species is determined by S-layer glycosylation patterns and the ups-pilin subunits (van Wolferen and Albers, unpublished). Additional studies on the molecular basis of this self-recognition will give more insights in how intraspecies communication is achieved and what are the barriers that drive speciation (Cadillo-Quiroz *et al.* 2012).

The *ups*-gene cluster is conserved among all Sulfolobales and encodes five proteins: UpsX, a hypothetical protein; UpsE, a secretion ATPase; UpsF, an integral membrane protein; and UpsA/B, two putative pilin subunits containing class III pre-pilin signal peptides (Figure 2B). Directly downstream to the *ups*-gene cluster, three genes are present encoding predicted DNA processing proteins. These are: an endonuclease III homologue, a ParB-like protein and an ATP-dependent helicase. Deletion of any of these genes in *S. acidocaldarius* results in lower survival rates upon UV treatment (van Wolferen and Albers, unpublished), we therefore speculate that they are involved either in the DNA uptake or the HR-mediated DNA repair pathway that is linked to the ups-system. A role in HR seems more likely as the helicase ortholog from *S. solfataricus* (Sso0112) was shown to catalyze the processing of Holliday junctions (Valenti *et al.* 2012).

Because type IV pili are often involved in the competence systems, one could speculate that uptake of DNA from lysed cells takes place rather than direct DNA exchange. However, the exchange of DNA was shown to be insensitive to DNAse treatment. Moreover, efforts to obtain recombinants by mixing cells with supernatant, lysed cells or marker DNA failed (Grogan 1996). This demonstrates that exchange of DNA indeed occurs directly from one living cell to another. Nevertheless, the mode of DNA transfer and the role of the ups-pili in this process are still unclear. One could imagine that the ups-pili are only involved in recognizing other cells from the same species and the initiation of a physical interaction. DNA transfer could subsequently occur via a yet unknown mechanism. Conjugation-like DNA transfer could for instance occur or cell fusion events might take place such as those observed during pNOB8 transfer (Schleper *et al.* 1995). However, when plating *ups*-gene inactivation mutants directly on top of each other, which theoretically would also bring cells in a close proximity, no recombinants can be obtained (Ajon *et al.* 2011). It can therefore be speculated that the ups-pili fulfill an active role in DNA exchange. The pili might function in a similar manner as type IV pili involved in natural transformation. In competent bacteria, however, retraction of the pili is almost always essential and in *Sulfolobus* species so far no retraction ATPase could be found. Furthermore, an unsolved puzzle is the directionality of the DNA transfer in *Sulfolobus* species. All cells

appear to be serving as donor and as recipient (Grogan 1996) what makes sense as all cells are genotypically similar. Following the DNA damage hypothesis, it would be most logical for a cell with damaged DNA to acquire DNA from another cell, possibly functioning as a template for HR. As it seems that there is little to no specificity in the chromosomal DNA that can be transferred between cells, it is hard to use chromosomal markers to study directionality in more detail.

Besides a role in cellular aggregation, ups-pili were also shown to be involved in biofilm formation (Koerdt *et al.* 2010). Moreover, ups-pili may play a role in HGT. Even though *Sulfolobus* species appear to aggregate only species specifically, in nature rare intra-species DNA exchange might have occurred, thereby promoting HGT. Overall, we can say that ups-mediated DNA transfer has proven to be a very interesting and unique mechanism of the Sulfolobales. So far, evidences in favor of a role in DNA repair are strong. Future studies might improve our understanding about intraspecies recognition, the mode of DNA transfer and confirm the function of DNA repair.

## SUMMARY AND CONCLUDING REMARKS

Transfer of DNA has been shown to occur in all domains of life although this process can take place by means of different mechanisms. The widespread occurrence stresses the universal significance of DNA transfer for life on earth. Here, we focused on why and how DNA is transferred among hyperthermophilic prokaryotes. DNA transfer at high temperatures may serve an important role in evolution. HGT has proven to be a powerful driving force for prokaryotic adaptation to changing environments such as rising temperatures. Moreover, as DNA degradation is increased at higher temperatures, specialized DNA repair mechanisms involving DNA uptake may play an important role in cell survival. A third but more controversial role of DNA uptake is the use of nucleotides as a nutrient source.

Numerous DNA transfer mechanisms have been described for hyperthermophilic microorganisms. Some are based on the direct contact of neighboring cells, such as conjugation and the production of nanotubes or UV-induced pili, others involve the direct uptake of DNA from the environment (natural transformation). Lastly, vehicles such as viruses or vesicles might function in DNA transfer. One could speculate that especially at higher temperatures, DNA transfer methods that involve direct cellular contact or DNA-protecting vehicles are favored. In that way, unprotected DNA is not exposed directly to the DNA damaging surroundings. Nevertheless, uptake of free DNA has been described for several (hyper)thermophilic microorganisms, including the *Thermus thermophilus* competence system. The latter mechanism is the most efficient competence system

found thus far in nature, which might relate to its proposed role in the adaptation to the high temperatures. In this respect, a high uptake rate also ensures limited exposure of free DNA to the surroundings.

Future research will undoubtedly expand the array of DNA transfer mechanisms among hyperthermophiles. Though more importantly, observed transfer processes need to be unraveled mechanistically, as their exact functioning is often still far from well understood. Interesting examples include conjugation and natural transformation among hyperthermophilic archaea which are both thought to differ extremely from their bacterial counterparts.

As genetic manipulation of hyperthermophilic bacteria and archaea has shown to be very challenging, further studies on their DNA transfer methods may advance the development of such tools. For example, the natural competence of *T. thermophilus* (Koyama *et al.* 1986), *T. kodakarensis* (Santangelo *et al.* 2008, 2010; Sato *et al.* 2003, 2005) and *P. furiosis* (Basen *et al.* 2012; Lipscomb *et al.* 2011) enabled the development of genetic tools. Moreover, the SSV1-based shuttle vector pMJ05 enabled reporter gene studies and the expression of proteins in *S. solfataricus* (Jonuscheit *et al.* 2003). Future studies will therefore not only increase our knowledge about the extremely diverse DNA transfer mechanisms functioning at high temperatures, but will also promote the study of other aspects in hyperthermophiles using these genetic systems.

## ACKNOWLEDGEMENTS

M.vW was supported by a grant from the German Science Foundation (DFG, AL1206/3-1). M.A was supported by an ALW grant from the Dutch Science Organization (NWO). S.V.A. received support from intramural funds of the Max Planck Society.

## SCOPE OF THE THESIS

*Sulfolobus* species are among the best characterized members of the archaeal domain. They belong to the kingdom of the Crenarchaeota, which mainly includes thermoacidophiles. Sulfolobales live in an extremely hot environment and grow optimally around 80°C at low acidic pH 2-3. Maintenance of the genomic integrity under such DNA damaging conditions is crucial for viability. However, while the archaeal information processing pathways are eukaryal-like, the DNA repair systems in archaea are still only poorly understood. Work described in this thesis is focussed on such DNA repair mechanisms, but in particular on the characterization of a novel chromosomal marker exchange system that is UV-induced. **Chapter 1** reviews our current insights on the mechanisms of DNA exchange in hyperthermophilic bacteria and archaea. **Chapter 2** identifies the pili biogenesis *ups*-operon (UV-inducible pili operon of *Sulfolobus*) that is expressed in response to UV irradiation in *Sulfolobus solfataricus*. This operon encodes type IV pili that are required for UV induced cellular aggregation. Deletion of the gene encoding the secretion ATPase of this assembly system abolished the UV dependent formation of pili as well as aggregate formation. Cellular aggregates could also be induced by agents that induce double-strand breaks in DNA. The *ups*-operon and the UV response in *Sulfolobus acidocaldarius* was further investigated in **Chapter 3**. Here species-specific aggregation was demonstrated and a UV-inducible DNA transfer process was characterized that is associated with this aggregation phenomenon. Using a mating assay, recombination rates were increased up to three orders of magnitude upon UV irradiation. Moreover, the presence of the ups-system increased the fitness and survival of UV-treated cells. Molecular analysis of the genes that are part of the UV-inducible pili operon from *S. acidocaldarius* was the subject of **Chapter 4**. The effect of the deletion of all individual *ups*-genes on pili formation, cellular aggregation and DNA transfer was analysed. *UpsA* and *upsB* both encode pre-pilin subunits and the deletion analysis demonstrates that both are required for efficient pilin formation, suggesting that the type IV pilus is composed of two subunits. This analysis also included the *upsX* gene that has an unknown function. UpsX was found to be involved in efficient DNA transfer, but is not essential for pili formation or cellular aggregation.

# 2

# UV-INDUCIBLE CELLULAR AGGREGATION OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS SOLFATARICUS* IS MEDIATED BY PILI FORMATION

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*Molecular Microbiology 2008 70(4):938-52*

## ABSTRACT

The hyperthermophilic archaeon *Sulfolobus solfataricus* has been shown to exhibit a complex transcriptional response to UV irradiation involving 55 genes. Among the strongest UV-induced genes was a putative pili biogenesis operon encoding a potential secretion ATPase, two pre-pilins, a putative transmembrane protein and a protein of unknown function. Electron microscopy and image reconstruction of UV-treated cells showed straight pili with 10 nm in diameter, variable in length, not bundled or polarized and composed of three evenly spaced helices, thereby clearly being distinguishable from archaeal flagella. A deletion mutant of SSO0120, the central type II/IV secretion ATPase, did not produce pili. It could be complemented by reintroducing the gene on a plasmid vector. We have named the operon *ups*-operon for UV-inducible pili operon of *Sulfolobus*. Overexpression of the pre-pilins, Ups-A/B (SSO0117/0118) in *Sulfolobus* resulted in production of extremely long filaments. Pronounced cellular aggregation was observed and quantified upon UV treatment. This aggregation was a UV-dose-dependent, dynamic process, not inducible by other physical stressors (such as pH or temperature shift) but stimulated by chemically induced double-strand breaks in DNA. We hypothesize that pili formation and subsequent cellular aggregation enhance DNA transfer among *Sulfolobus* cells to provide increased repair of damaged DNA via homologous recombination.

## INTRODUCTION

The ability of *Bacteria* and *Archaea* to form multicellular structures is observed in a variety of biological systems. This fascinating phenomenon of a collective behaviour can be manifested in the formation of biofilms from mixed microbial mats, cellular aggregates or microcolonies. Multicellular structures represent an essential strategy for adaptation to changing environmental conditions or even survival (Shapiro 1998; Davey and O'Toole 2000; Battin *et al.* 2007). Cells organized in biofilm-like structures show a higher resistance to toxic compounds, as for example antimicrobials (Patel 2005) or to physical stress, like shifts in temperature or pH, or exposure to UV light (Ojanen-Reuhs *et al.* 1997; Roine *et al.* 1998; Elasri and Miller 1999; Martinez and Casadevall 2007). In addition, microorganisms benefit from the attachment on substrates like, e.g. suspended particles, which provides a higher nutrient availability (Davey and O'Toole 2000). Also genetic transfer, i.e. DNA exchange via conjugation, plays an important role in biofilms to disseminate specific genes of metabolic pathways (Gasson and Davies 1980; Molin and Tolker-Nielsen 2003). The rate of conjugative DNA exchange in biofilm structures is enhanced and conjugative pili stabilize the biofilm structure (Gasson and Davies 1980; Ghigo 2001; Molin and Tolker-Nielsen 2003; Reisner *et al.* 2006).

Cellular aggregation is mainly reported for organisms of the domain *Bacteria*, while comparably few but quite diverse examples have been found in the domain of the *Archaea.* In anoxic sediments of the ocean anaerobic methane-oxidizing archaea form synergistic communities with sulphate-reducing bacteria in the form of structured consortia (Boetius *et al.* 2000). An unusual microbial community organized in string-of-pearls was found in cold sulphurous water. It is formed by the euryarchaeon SM1 that grows in close association with the bacterium *Thiothrix* sp. and forms complex and unusual cellular appendages (Moissl *et al.* 2003, 2005). Single-strain cultures of the hyperthermophilic euryarchaeote *Archaeoglobus fulgidus* form a protein-, metal- and polysaccharide-containing heterogeneous biofilm, which is inducible by various environmental stressors (LaPaglia and Hartzell 1997). *Pyrococcus furiosus* can form surface attached microcolony structures mediated by multifunctional flagella, which can also form cable-like structures to mediate cell–cell contacts (Näther *et al.* 2006; Schopf *et al.* 2008).

Beside adherent multicellular structures that are found attached to diverse surfaces, non-adherent floating multicellular structures are also described for archaea. *Methanosarcina mazei*, for example, forms aggregates during exponential growth (Mayerhofer *et al.* 1992) and halophilic archaea do so in the presence of divalent cations (Kawakami *et al.* 2005, 2007). For the halophilic euryarchaeote *Halobacterium volcanii* and the hyperthermophilic crenarchaeote *Sulfolobus*

sp. cellular aggregation was observed in context with conjugative DNA transfer (Rosenshine *et al.* 1989; Schleper *et al.* 1995).

Characteristic for all types of cellular aggregation is the attachment between single cells, mostly mediated or stabilized by exopolysaccharides (EPS) and/or proteins as was shown for many bacterial systems (Davey and O'Toole 2000; Klemm *et al.* 2004; Kawakami *et al.* 2007). Some microorganisms like *Xanthomonas* and *Pseudomonas* use type IV pili to initiate or mediate the cellular aggregation (Ojanen-Reuhs *et al.* 1997; Bhattacharjee *et al.* 2001). A *Pseudomonas aeruginosa* mutant defective in the type IV pilus biogenesis was unable to attach on surfaces and form microcolonies (O'Toole and Kolter 1998; O'Toole *et al.* 2000).

The type IV pili biogenesis pathways of bacteria are not only closely related to the type II protein secretion systems (Sauvonnet *et al.* 2000; Köhler *et al.* 2004), but also to the archaeal flagella systems. This was shown by bioinformatic and biochemical analyses (Faguy *et al.* 1994; Bardy and Jarrell 2002; Peabody *et al.* 2003). In addition, it has been shown that the flagella of *Halobacterium salinarum* and *Sulfolobus shibatae* are in symmetry and structure more closely related to the bacterial type IV pili than to bacterial flagella (Cohen-Krausz and Trachtenberg 2002, 2008). The core components of the bacterial and archaeal systems are: (i) a type II/IV secretion system ATPase, (ii) a multispanning transmembrane protein, and (iii) the pre-pilin-like proteins with a characteristic N-terminal signal sequence (termed class III signal peptides) that form the structure of the pilus (Peabody *et al.* 2003). In the genome of the crenarchaeote *Sulfolobus solfataricus* three putative type IV pili loci were identified (Albers and Driessen 2005). The operon SSO2316 (named after the central ATPase) codes for the flagellum of *S. solfataricus* (Szabó *et al.* 2007a). The operon SSO2680 encodes a recently described bindosome assembly system (Bas) needed for the functional surface localization of sugar-binding proteins (Zolghadr *et al.* 2007). The biological function of the third operon SSO0120, spanning ORFs *sso0117* through *sso0121*, was unclear. Using whole-genome microarray studies to analyse the UV response of *S. solfataricus* we observed that the genes *sso0117* to *sso0121* were among the most highly induced genes using a UV dose of 75 $J/m^2$ at 254 nm (Fröls *et al.* 2007). A strong upregulation of the operon was also observed by an independent study of White and co-workers using a higher UV dose of 200 $J/m^2$, with *S. solfataricus* and *S. acidocaldarius* (Dorazi *et al.* 2007; Götz *et al.* 2007). In parallel to the strong transcriptional UV response we observed a massive aggregation of the cells, which disappeared after regeneration (Fröls *et al.* 2007).

In this study we demonstrate that extracellular pili-like structures, thinner than flagella, are formed upon UV light treatment. They are encoded by the UV-inducible (type IV-like) pili operon, as shown by targeted gene knockouts. Furthermore, we show that these pili structures are essential for the UV-dependent

auto-aggregation of *S. solfataricus* cells and that this phenomenon is driven by double-strand breaks (DSB) in the DNA, but not by other stressors, such as pH or temperature shifts.





## RESULTS

### UV-inducible expression of the *sso0117–sso0121* gene cluster

The induction of the genes *sso0118* and *sso0117* occurred as one of the strongest and fastest transcriptional reactions detected in an earlier genome-wide microarray study on the impact of UV light exposure of *S. solfataricus* cells (Fröls *et al.* 2007). These genes belonged to a cluster, and possibly an operon of five genes (*sso0117* through *sso0121*) all of which were strongly induced with a maximal induction of 14-fold for *sso0118* (Fröls *et al.* 2007). The transcriptional increases were observed at 1.5–5 h after UV treatment. Over the time-course of 8.5 h, a similar transcriptional reaction pattern for these genes was observed, but not for the upstream or downstream flanking genes (*sso0116* and *sso0115*, *sso0122*). This indicated transcription from a common promoter, as suggested earlier under non-inducing growth conditions (Albers and Driessen 2005). Only the gene *sso0118* deviated from this UV-dependent pattern and was up to 3.5-fold higher induced, which may indicate the presence of an additional promoter or alternatively, a higher stability of the transcript.

Bioinformatic analysis indicated a putative type IV pili biogenesis operon, represented by a type II/IV secretion system ATPase (SSO0120) and an integral membrane protein (SSO0119) (Figure 1A). The deduced protein sequence of the ATPase SSO0120 contained Walker A/B sites and a VirB11-related ATPase conserved domain (COG630N). The protein belonged to the TadA subfamily of type IV ATPases (Planet *et al.* 2001). SSO0119 contained nine predicted transmembrane helices and a TadC (COG2064N) conserved domain. Thus both proteins were homologous to factors of the Tad system (TadA and TadB/TadC), which conveys non-specific tight adherence of *Actinobacillus* on surfaces (Kachlany *et al.* 2001). The SSO0118 and SSO0117 proteins harboured an N-terminal class III secretory signal sequence as found in type IV pilin precursors. No functional predictions could be made for the first gene, *sso0121*, which encoded a highly hydrophilic protein exclusively found in the genomes of *Sulfolobales.*

The putative pili operon was well conserved in the order *Sulfolobales*, with the same gene arrangement in the strains *Sulfolobus tokodaii* and *S. acidocaldarius* (Table S2 and Szabó *et al.* 2007b). Further similarities were only found to genes of the hyperthermophilic crenarchaeon *Metallosphera sedula*, belonging to a closely related order. In *M. sedula*, homologues of *sso0120*, *sso0119* and *sso0117* form an operon structure whereas *sso0118* is located in a different genomic region.

## Maturation of pre-pilins

Both SS0117 and SSO0118 encode predicted proteins of 15 and 16 kDa, respectively. They contain a signal sequence with the predicted cleavage site for the type IV pre-pilin peptidase PibD (Albers *et al.* 2003; Szabó *et al.* 2007b). In SSO0117 and SSO0118 only 6 and 16 amino acids would be cleaved by PibD respectively (Figure 1B). The ORFs of SSO0117 and SSO0118 were cloned into an *Escherichia coli* expression vector already containing PibD (Szabó *et al.* 2007b). Using the *in vivo* assay the expression of the pre-pilin proteins was induced for 2 h before the expression of the peptidase was induced. Western blot analysis of crude membrane extracts of the recombinant *E. coli* cells showed that SSO0118 was processed by PibD resulting in a faster running protein species when compared with the full-length protein (Figure 1C, lanes 2 and 3). Cleavage of SSO0118 was already observed before induction of the expression of PibD, most likely because the promoter used for the expression is leaky and the enzyme cleaves the substrate very efficiently. Cleavage of the signal peptide of SSO0117 could not be observed,

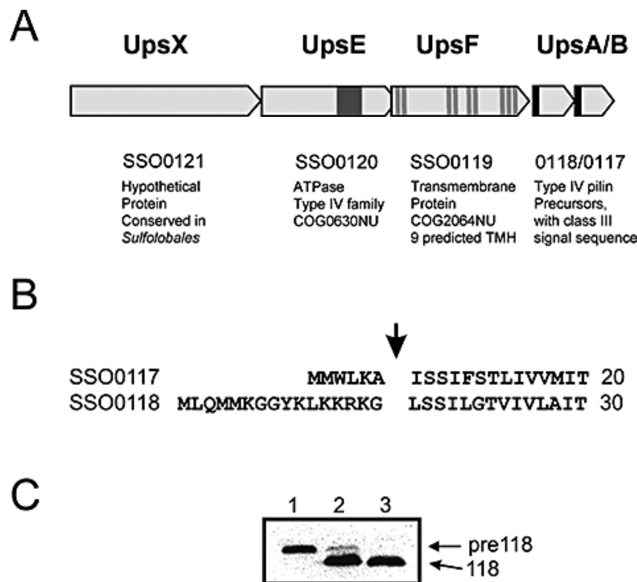


**Figure 1.** A. Composition and characteristics of the *ups*-operon (UV-inducible pili operon of *Sulfolobus*). Transcriptional induction after UV treatment has been shown in Fröls *et al.* (2007). TMH, transmembrane helices. B. N-termini of UpsA/B (SSO0117/0118) with processing site of PibD indicated by an arrow. C. Result of *in vivo* cleavage assay of SSO0118-HA by PibD in *E. coli*. Expression of the protein was detected by Western blot analysis using HA-tag antibodies. Lane 1: expression of SSO118-HA in the absence of PibD; lane 2: 2 h after arabinose induction; lane 3: 2 h after induction of PibD by IPTG.

most probably because the difference between the precursor and the processed form do not differ enough to be separated on SDS-PAGE. Experiments to separate these two forms in isoelectric focusing gel electrophoresis failed.





**UV-induced pili formation**

To identify pili, cells were analysed by electron microscopy after UV treatment. To exclude that any extracellular structures were not artefacts of flagella we used the *S. solfataricus* knockout strain Δ*flaJ* that does not produce flagella (Szabó *et al.* 2007a). Only on the surface of the UV-treated cells, pili-like structures were observed (Figure 2A). These pili structures were spread over the whole surface and were not polarized at one cell side. Most of the cells of a UV-treated culture contained many pili, some had less or very few (only two to three pili), and few cells did not express pili on their surfaces at all. A time-course experiment showed that the first pili-like structures were observed at 1 h after UV treatment.

In comparison with the flexible flagella, the pili showed a more straight and rigid structure. Pili of up to 16 μm in length or even longer were observed. However, such long filaments where only found detached from the cells, which indicated that they are more fragile than flagella. Because the pili appeared straight for most of their length, it was possible to process them by single-particle analysis selecting straight segments of almost up to 100 nm. About 700 segments were extracted from long pili, aligned and averaged. The final average projection map is shown

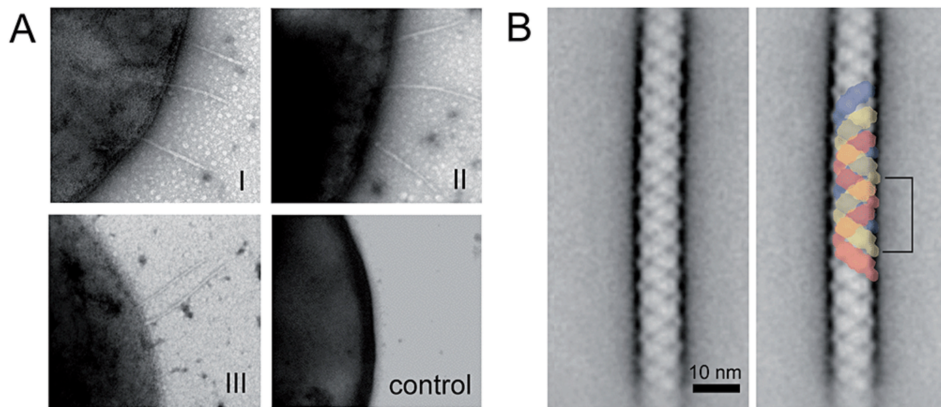


**Figure 2.** Electron microscopic analysis of UV-inducible pili in *S. solfataricus*. A. Δ*flaJ* cells were analysed by electron microscopy 3 h after UV light treatment (I, II and III) and mock treatment for the control. B. Image processing of pili. Left: projection map obtained after processing 700 non-overlapping fragments of straight pili. Right: Scheme of three-stranded helical arrangement of the pili overlayed. The horizontal lines indicate the pitch of the structure which is 15.5 nm.

in Figure 2B. The structure appeared to consist of three evenly spaced helices. The pitch (repeating unit) of the pili was 15.5 nm and the maximal diameter was about 10 nm. In the negatively stained samples the single helices appeared almost uniformly stained without any clear density differences that could give clues about the handedness (left- or right-handed) of the helices.





**Cellular aggregation after UV treatment**

The appearance of pili upon UV treatment that could mediate cell-to-cell contacts inspired us to analyse microscopically the formation of cell aggregates (Fröls *et al.* 2007). We have shown earlier that aggregation occurs with high frequency independent of the *S. solfataricus* genotypes, because experiments with four different strains [P1, PH1, PH1-M16, PH1(SSV1)] showed the same phenotypic reaction. With increasing time after UV treatment, an increasing number of cells were found in aggregates with the highest amount of aggregation found at 6–8 h after UV treatment (Fröls *et al.* 2007 and Figure 6). The aggregates increased also in size. While three to five cells were found in the early aggregates, bigger complexes formed at later time points. The shape of the early aggregates seemed to be random, as variations of pyramids, circle shapes, straight and branching chains were observed (not shown). In the later stages (6 h) the cells accumulated to big clusters of > 100 cells. As it was impossible to count the number of cells in such aggregates, our quantitative data (% cells in aggregates of total cell count) generally represent an underestimate.

Attempts to destroy the cell–cell connections by shear force experiments resulted in cell lysis at all stages but not in disaggregation, indicating a high stability of the aggregates. The induction of cellular aggregation was UV-dose-dependent (Figure 3). We treated the cells with seven different UV doses ranging from 5 to 1000 $J/m^2$. Growth retardation of the respective cultures was directly proportional to the applied UV dose (data not shown). The highest cellular aggregation was observed 6 h after UV treatment, i.e. at the expected maximum. The highest amount of cellular aggregation was found with 75 $J/m^2$ (up to 50% and sometimes even 70% of cells in aggregates, Figure 3A and Fröls *et al.* 2007) and with 50 $J/m^2$ (at least 40% of the cells were in aggregates of ≥ 3 cells, Figure 3A). Even the lowest dose of UV light of 5 $J/m^2$ induced the cellular aggregation, whereas the high UV doses of 200 and 1000 $J/m^2$ showed a very low and no significant aggregation reaction respectively. We also observed a strong correlation between the size and amount of cellular aggregates (Figure 3B). Low doses of 5 and 10 $J/m^2$ resulted in cellular aggregates of < 7 cells. Only upon a UV dose of 50–75 $J/m^2$, large aggregates of 10–20 cells or many more were generated frequently. Because cells in these biggest aggregates could not be counted, our numbers in Figure 3A represent an underestimate for 50 and 75 $J/m^2$. In the case of the high UV doses of 200 and 1000 $J/m^2$ no aggregates > 4 cells were observed.

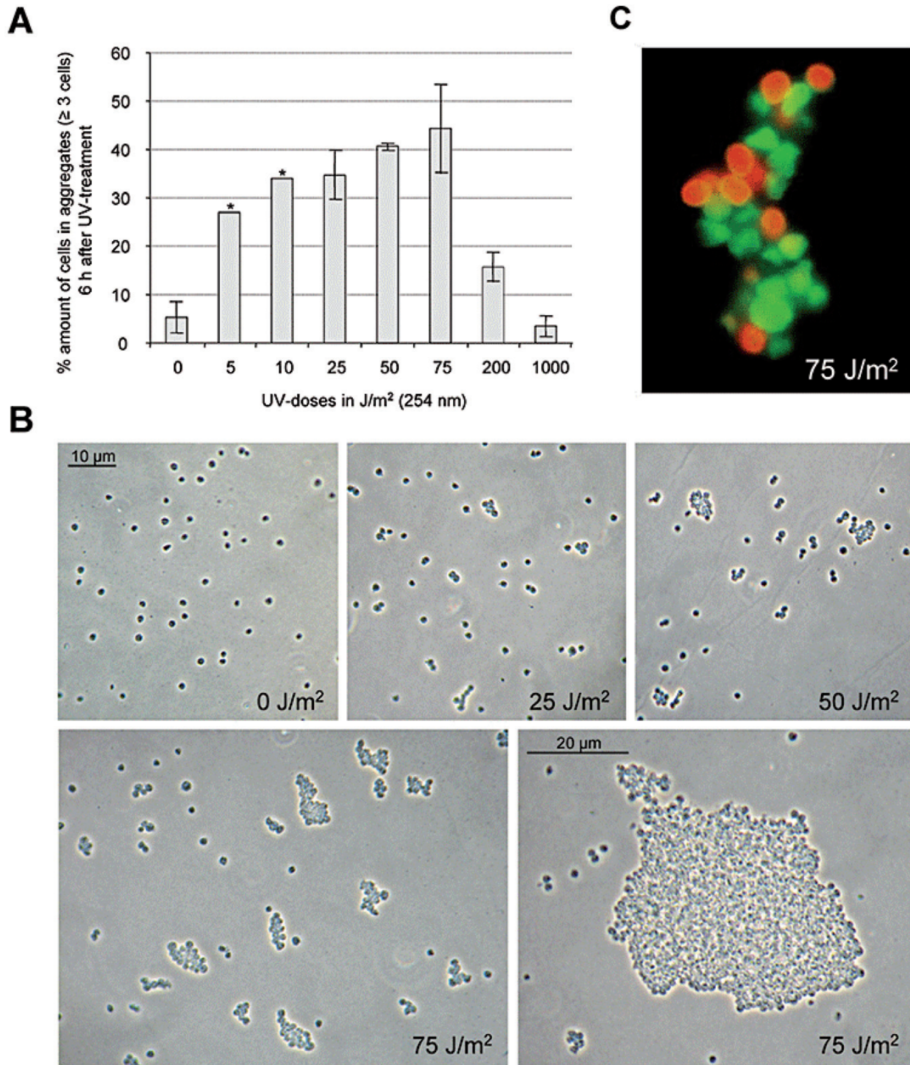


**Figure 3.** Aggregation of *S. solfataricus* cells after treatment with different UV doses. A. Quantitative analysis of cellular aggregation at 6 h after UV treatment. Exponential cultures were treated with 0, 5, 10, 25, 50, 75, 200 and 1000 J/m$^2$. The percentage amount of cells in aggregates (≥ 3 cells) is given in relation to the total cells. For each UV dose the amount of cells in and outside aggregates were counted until 500 single cells were found. The bars display the mean of three independent experiments, except for 5 and 10 J/m$^2$ (see asterisk), where only one experiment was performed. B. Light micrograph of *S. solfataricus* cell aggregates at 6 h after UV treatment with different UV doses. The size of the aggregates increased with the UV dose; the biggest aggregates were found after treatment with 50 and 75 J/m$^2$. C. Fluorescence micrograph of a *S. solfataricus* cell aggregate at 6 h after UV treatment at 75 J/m$^2$. Cells were stained with the LIVE DEAD Baclight (Invitrogen) assay. Living cells are labelled in green and dead cells in red. Big aggregates of > 20 cells were mostly found at 3 h after treatment. For quantitative analysis of the cell vitality at different UV doses see Table 1.

By using different vital staining techniques (see *Experimental procedures*), we investigated whether dead cells accumulate in aggregates (Table 1). In the case of the lowest UV dose of 5 J/m$^2$ only 8% of the total cells in aggregates (≥ 3 cells) were dead. The amount of dead cells increased proportionally with the UV dose but was far lower than the number of living cells. The majority of the cells (64%) present in the infrequent aggregates at 200 J/m$^2$ were not alive. At lower UV dose, like 75 J/m$^2$, even large aggregates of > 20 cells were almost uniformly composed of living cells (Figure 3C).





**Table 1.** Cell vitality of *S. solfataricus* in aggregates formed after treatment with UV light.

| UV dose (J/m$^2$) | Vital cells in%[a] |
|---|---|
| 5 | 92 |
| 10 | 88 |
| 25 | 83 |
| 50 | 66 |
| 75 | 56 |
| 200 | 36 |

a. At 6 h after UV treatment a live and dead stain was performed (see Experimental procedures and also Fig. 3C).
A minimum of 50 aggregates ≥ 3 cells were counted per each UV dose and the fraction of vital cells is given in relation to the total cells found in aggregates.

**Gene knockout in the UV-inducible pili operon abolishes pili formation and cellular aggregation**

To prove that pili were indeed assembled from components expressed from the putative pili operon, a deletion mutant was constructed in which the ATPase (*sso0120*) was replaced by insertion of a *lac*S cassette containing the *lacS* gene with its natural promoter and terminator region via a double cross-over. The successful deletion of the *sso0120* gene was confirmed by Southern analysis and RT-PCR (Figure 4A and B). The RT-PCR showed that under inducing conditions the *sso0120* mRNA was absent, while the downstream genes of the operon were still expressed, probably because of read through of the *lac*S terminator by the RNA polymerase. After UV treatment of the mutant Δ*sso0120* we could not observe any pili-like structures on the cellular surfaces by electron microscopy (Figure 4C, Ia and Ib). As control, we used the parent strain PBL2025 (Schelert *et al.* 2004) which clearly showed pili-like structures beside the flagella upon exposure to UV light (Figure 4C, IIa and IIb). When the mutant strain Δ*sso0120* was complemented with pSVA99 containing *sso0120* under the control of the *araS* promoter, pili were again

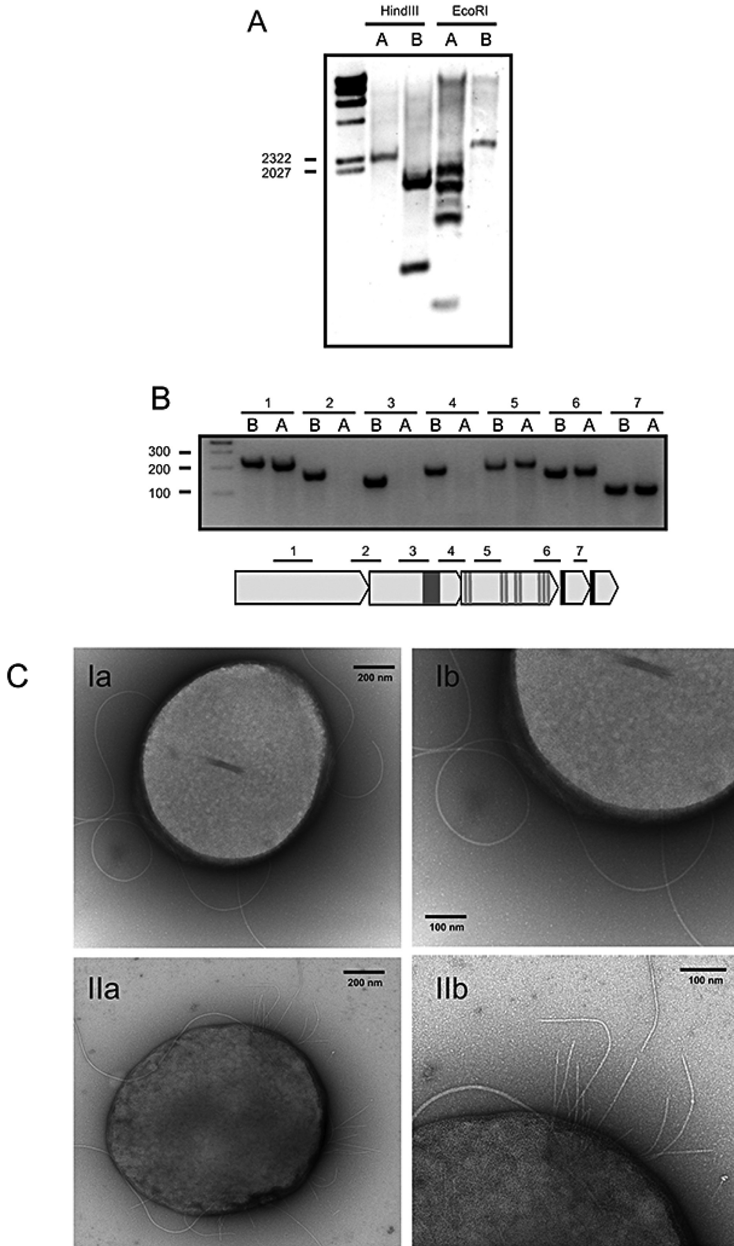


**Figure 4.** Analysis of the *sso0120* knockout strain. A. Southern blot analysis of wild-type PBL2025 (B) and the Δ*sso0120* (A) strain. Genomic DNA was each digested with HindIII or EcoRI respectively. B. RT-PCR analysis of PBL2025 (B) and Δ*sso0120* (A) strain after UV stress. The position of the primers used for the PCR reactions are indicated by the same number above the gel and the map of the operon. C. Electron micrographs 3 h after UV treatment. Only long flagella but no pili can be observed in the Δ*sso0120* strain (Ia and b) while the surface *of* PBL2025 (IIa and b) is covered with pili.

observed after UV treatment, but only when the cells were additionally incubated with arabinose (Figure 5A). Overexpression of both pilin genes, *sso0117* and *sso0118*, in the Δ*flaJ* strain using the virus-based vector construct pSVA96 resulted in the assembly of fewer, but extremely long and irregular pili (Figure 5B). The overexpression of the whole operon, *sso0121-0117*, using pSVA125 led to the formation of pili around the whole cell surface (Figure 5C), whereas the expression of a cytoplasmic control protein from pSVA31 did not result in the assembly of surface structures (data not shown). Together these data demonstrate that formation of the pili is dependent on expression of *sso0120* and that the two pre-pilins (or one of them) most likely form the subunits of the UV-inducible pili.

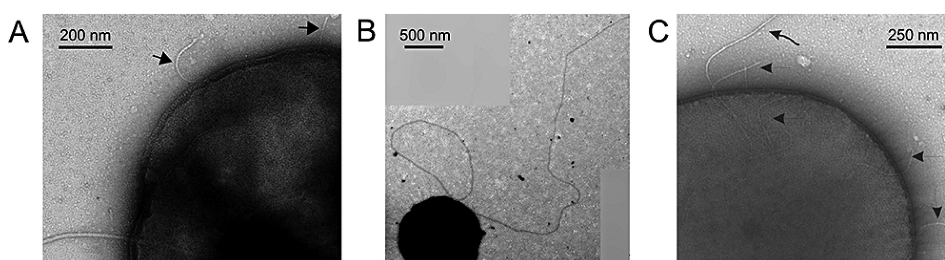


**Figure 5.** Electron micrographs of *S. solfataricus* cells assembling pili. (A) shows a UV-treated Δ*sso0120* cell expressing *sso0120* under the control of the *araS* promoter. (B) depicts a Δ*flaJ* cell overexpressing the pilins SSO0117/0118 and overexpressing the whole operon comprising SSO0121-0117 (C). In (B) two pictures were assembled to show the length of the pilus; in (A) and (C) pili are indicated by the arrows.

The Δ*sso0120* strain was further tested for its ability to form cellular aggregates upon UV exposure. After a treatment with a UV dose of 50 J/m$^2$ no significant cellular aggregation of more than four cells was observed (Figure 6). The amount of cells in aggregates accounted for less than 10% in the UV-treated culture and the control (mock-treated) culture, similar to the amount of cells in aggregates observed for the mock-treated cultures of the other four tested *S. solfataricus* strains. The *S. solfataricus* strain P1 and PH1-M16 (P1 Δ*lacS*) showed a maximum aggregation at 6–8 h after treatment, with an average of 45–50% cells in aggregates. In the same experiment, the PBL2025 and the Δ*flaJ* strains exhibited a shifted maximum at 8–10 h and a lower amount of aggregation with an average of 20%. The weaker reaction is most probably due to the different genotypes of these strains, which stem from PBL2025, an isolate from Yellowstone National Park *S. solfataricus* 98/2 (Schelert *et al.* 2004). Comparable results were observed when using a lower UV dose of 25 J/m$^2$ (Figure S1). Again, no significant cellular aggregation was observed for strain Δ*sso0120*. The P1 and PH1-M16 strains showed a lower amount of aggregation

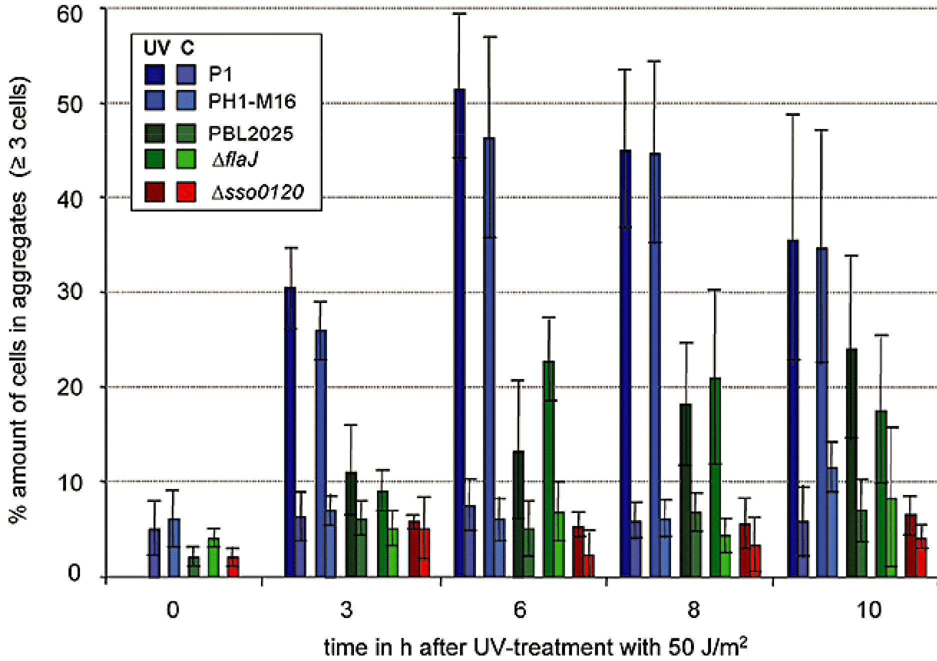


**Figure 6.** Quantitative analysis of the UV-induced cellular aggregation of different *S. solfataricus* strains at 0–10 h after UV treatment (UV) and mock treatment (C). The graph is based on four independent UV experiments for each strain. Cellular aggregation was observed at 3, 6, 8 and 10 h after UV treatment with 50 J/m² (254 nm). The bars display the percentage amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells (500–1000 single cells were counted). No UV-induced cellular aggregation was observed in the knockout strain Δ*sso0120*. Similar results were observed by using a UV dose of 25 J/m² (254 nm) displayed in Fig. S1

with 30–40%, as expected in relation to the lower UV dose. The amount of cells in aggregates in the case of the PBL2025 strain stayed the same, whereas with the Δ*flaJ* strain the amount of cells in aggregates increased to > 30% and the maximum shifted to 6 h. Based on these results we conclude that the UV induction of the putative pili operon, the inducible pili production and the cellular aggregation are functionally linked to each other. We therefore named the newly identified operon UPS for UV-inducible (type IV-like) pili operon of *Sulfolobus*, represented by the genes *upsX*, *upsE* (ATPase), *upsF* (TM protein), *upsA* and *upsB* (pre-pilins).

### Cellular aggregation is not inducible by other environmental stressors or in late growth phases

To analyse if cellular aggregation can be induced by conditions other than UV exposure, four strains that harbour the wild type of the *ups*-operon were used:

*S. solfataricus* strains P1, PH1-M16, PBL2025 and Δ*flaJ*. We monitored and quantified the extent of cellular aggregation after a temperature shift from 78°C to 88°C (heat shock) and down to 65°C (Kagawa *et al.* 2003), which corresponded to non-lethal heat- and cold-shock conditions that might be often encountered in hot springs. Shifts from pH 3 to 4 and down to pH 2.5 were similarly investigated. No significant cellular aggregation was observed under the tested conditions in any of the four tested strains. The amount of cells in aggregates (≥ 3 cells) was always below 10% (Figure S2). We also monitored the extent of cellular aggregation in the late growth phases of the cultures, from stationary to dead phase. Only at the beginning of the late stationary phase, i.e. at the start of growth retardation, a slightly increased cellular aggregation was noted. For strain P1 up to 24% of the cells were found in aggregates of four to seven cells at most, while the amount of cells in aggregates (≥ 3 cells) were lower than 10% in all other growth phases (Figure S2).

**Cellular aggregation is induced by treatment with DNA double-strand breaking-inducing agents**

As a response of *S. solfataricus* to UV light we observed earlier the formation of DSB in the genomic DNA (Fröls *et al.* 2007). Whereas *cis*-syn-cyclobutane pyrimidine dimers (CPDs) represent direct DNA damages caused by the UV light effect, DSB are probably formed as a result of collapsing replication forks at unrepaired sites in the genomic DNA. It has been speculated earlier that DSB might represent an intracellular signal for further cellular reactions. Therefore we determined whether the formation of DSB is connected to the formation of cellular aggregates. The induction of cellular aggregation of the different *S. solfataricus* strains P1, PH1-M16, PBL2025 and Δ*sso0120* in response to the DSB-inducing agents bleomycin (3 μg ml$^{-1}$) (Figure 7) and mitomycin C (5, 10 and 15 μg ml$^{-1}$) was investigated (Cannio *et al.* 1998; Reilly and Grogan 2002; Kosa *et al.* 2004) (Table 2). The concentrations we applied were non-lethal to the cells as investigated by plating efficiencies and growth behaviour in liquid cultures (data not shown). Cellular aggregation was monitored at 3, 6 and 8 h after the treatment and with bleomycin additionally at 1 and 10 h. All tested strains, except for the Δ*sso0120* strain, showed a significant cellular aggregation in response to the agents. Eight hours after the treatment with bleomycin, strains P1, PH1-M16 and PBL2025 exhibited 25–35% of cells in aggregates (Figure 7), while aggregation in the mock-treated cultures and the bleomycin-treated strain Δ*sso0120* remained below 10%. Similarly, although less strongly, mitomycin C induced aggregate formation in the *ups*-operon containing wild-type strains (P1, PBL2025), but not in the knockout strain (Table 2). These observations indicate that DNA damage and in particular DSB might be a direct or indirect signal for inducing aggregate formation.

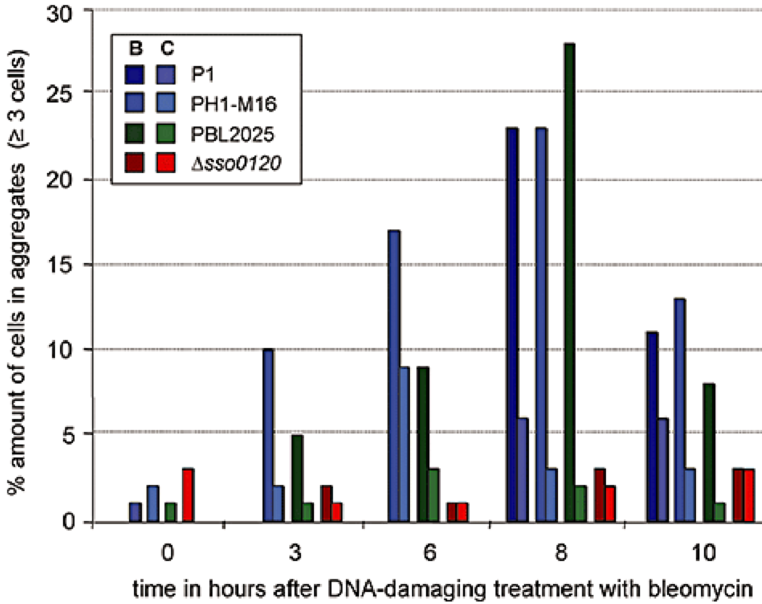


**Figure 7.** Aggregate formation of different *S. solfataricus* strains after treatment with bleomycin (B) (3 µg $ml^{-1}$) and mock treatment (C). No significant cell aggregation was observed with the knockout strain Δ*sso0120*. The bars display the percentage amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells (500 single cells were counted).

**Table 2.** Cellular aggregation in percentage after treatment with mitomycin C[a].

| | | Time in hours after treatment | | |
|---|---|---|---|---|
| **Strain** | **Mitomycin C dose** | **3 h** | **6 h** | **8 h** |
| P1(wild-type strain) | 5 µg $ml^{-1}$ | 5 | 10 | 10 |
| | 10 µg $ml^{-1}$ | 8 | 10 | 16 |
| | Control | 1 | 2 | 2 |
| PBL2025 | 5 µg $ml^{-1}$ | 1 | 10 | 9 |
| | 15 µg $ml^{-1}$ | 6 | 12 | 10 |
| | Control | 1 | 1 | 3 |
| PBL2025:Δ*sso0120* | 5 µg $ml^{-1}$ | 1 | 1 | 2 |
| | 15 µg $ml^{-1}$ | 0 | 0 | 0 |
| | Control | 0 | 1 | 0 |

a. The percentage amount of cells in aggregates (≥3 cells) in relation to the total number of evaluated cells is given (a minimum of 500 single cells were counted).

## DISCUSSION

The special living conditions of *Archaea* in extreme environments make them interesting objects to study adaptations and stress responses. In particular hyperthermophilic and acidophilic *Archaea* like *S. solfataricus* have to deal with a constant stress and DNA damage in their harsh environments. Here we present the identification and characterization of an archaeal pili system that mediates cellular aggregation of *S. solfataricus* in response to UV damage. The genes encoding the now called *ups*-operon for UV-inducible (type IV-like) pili operon of *Sulfolobus* had earlier been identified to be UV-dependently induced in a genome-wide DNA microarray analysis (Fröls *et al.* 2007).

To our knowledge this is the first reported study on a UV-inducible pili-mediated auto-aggregation system. As discussed below, its induction seems to be coupled to the DNA DSB caused by UV irradiation. We suspect that cellular aggregation mediates DNA repair via a conjugation-like process, as an enhanced exchange of chromosomal markers has been observed upon UV irradiation as well as increase in the transcripts levels of genes involved in homologous recombination.

**UV-inducible pili mediate cellular aggregation**

By electron microscopic analysis we found a strong correlation between the formation of extracellular pili on the cellular surface after UV treatment and the expression of the *ups*-operon, both of which appeared first at 1 h after UV treatment and reached a maximum within 5–6 h. To test our hypothesis that the gene products of the *ups*-operon are responsible for the production of the pili we used the recently developed genetic system (Albers and Driessen 2007) to produce a specific knockout of the putative secretion ATPase UpsE (SSO0120). No pili structures were observed on the cellular surface of the Δ*sso0120* strain. By testing this strain in a quantitative cellular aggregation analysis, we proved that the pili are necessary for the cellular aggregation of *S. solfataricus* after UV treatment. Cellular aggregates were as infrequent (i.e. lower than 10% of all cells) as in mock-treated controls of four different *S. solfataricus* strains. Image analysis of isolated pili structures showed that the pili are much thinner in diameter and clearly distinguishable from the flagella of *S. solfataricus* (Szabó *et al.* 2007a). A detailed structural analysis of another archaeal pilus has recently been published from a methanogenic euryarchaeote *Methanococcus maripaludis* (Wang *et al.* 2008).

The pili-like structures of *S. solfataricus* are spread over the whole cellular surface. They are not bundled or polarized like the cable-like flagellar bundles of *P. furiosus*, which mediate cell attachment (Näther *et al.* 2006) or the type IV pili of the Tad system from *Actinobacillus* species that mediate non-specific adherence (Kachlany *et al.* 2000, 2001). Experiments to disconnect cellular aggregates by

shearing forces failed, indicating that the cell–cell contacts were highly stable once formed and showing that the cellular aggregates were not a result of an unspecific accumulation. The latter was also ruled out by a live and dead stain analysis (Figure 3C and Table 1). The detailed mechanisms of auto-aggregation is, however, still unknown. It has been reported that the bacterial type IV pili are bound with their tip on surface structures or other cells (Mattick 2002). So far, we did not observe any attachment to surfaces. However, our experiments were performed under moderate shaking in glass flasks, such that one cannot rule out the possibility of surface attachment under different conditions.

**UV-inducible cellular aggregation is highly dynamic**

A quantitative assay was developed in this study to analyse the dynamics of cellular aggregation in more detail. We showed that the aggregation is a fast process induced by the UV-dependent reaction of *S. solfataricus* and seems to occur in two phases. First, small aggregates of three to five cells accumulate, which later aggregate to larger forms. The maximum of aggregation was reached at 6–8 h after UV treatment, followed by a clear disappearance, interpreted as an active disaggregation. One has to note that the absolute amount of cellular aggregation is by far underestimated because cell aggregates of more than 20 cells were uncountable and the biggest aggregates with even up to 100 and more cells were found frequently at 6 h after UV treatment. Furthermore, cell aggregates of two were not incorporated in the calculations in order to exclude dividing cells.

In correlation to the cell cycle length of *S. solfataricus*, which is around 7 h, the dynamics of this process are relatively fast. For example, the cellular packets of *M. mazei* need 2–6 days to form the lamina structures, and then remain stable over 6–11 days until the culture reaches stationary growth phase and the lamina disaggregate (Mayerhofer *et al.* 1992). The stress induced biofilm formation of *A. fulgidus* occurs in 2–12 h, but in this case no disaggregation was reported (LaPaglia and Hartzell 1997).

**UV light is the only identified stressor to induce auto-aggregation**

It is reported that cells organized in multicellular structures show a higher resistance to different environmental stressors, like temperature, pH and also UV light (Ojanen-Reuhs *et al.* 1997; Roine *et al.* 1998; Martinez and Casadevall 2007). Treatment of the hyperthermophilic archaeon *A. fulgidus*, with a high dose of UV light and other physical or toxic stressors, results in a biofilm production (LaPaglia and Hartzell 1997). Mutants of plant pathogen bacteria *Pseudomonas syringae* and *Xanthomonas campestris* defect in the auto-aggregation showed a higher sensibility to UV irradiation than the wild type capable of forming multicellular structures (Ojanen-Reuhs *et al.* 1997; Roine *et al.* 1998). A decreased sensitivity to UV light

and other environmental stressors was also reported for biofilms of the yeast-like fungi *Cryptococcus neoformans* (Martinez and Casadevall 2007). None of the stressors that we used for *S. solfataricus* induced cellular aggregation, nor did late growth phase stages. This stands in contrast to all given examples of multicellular structures, which are typically interpreted as an advantageous life form under harsh or specialized environmental conditions. Thus *S. solfataricus* shows a unique multicellular formation which is not a general effect of a stress response.

Interestingly, the extent of cellular aggregation (aggregate sizes and the number of cells involved) was dependent on the UV dose. Relatively high doses of UV light, like 200 or 1000 J/$m^2$ resulted in an insignificant amount of small aggregates (≤ 4 cells) and killed most of the cells. In contrast a relatively low dose of UV light, like 5 J/$m^2$, induced cellular aggregation. In nature sunlight composed of up to about 96% UV-A reaches the ground with *c*. 4% UV-B that is the most DNA-damaging factor. The daily dose of DNA-damaging UV-B light on a sunny day in the northern and southern world hemispheres is measured between 1000 and 3000 J/$m^2$ over 24 h (depending on the season). The experimentally used UV-C (254 nm) is about 100-fold more effective than UV-B in inducing CPDs (Kuluncsics *et al.* 1999). With reference to the observation that even low dose of UV light significantly induces the cellular aggregation of *S. solfataricus* we conclude that this phenotypic effect reflects the behaviour of the organism to the sunlight in the natural environment.

**Cellular aggregation is induced by DNA DSB and might mediate a recombinational repair among chromosomes of mating cells**

Between 2 and 8 h after UV treatment we observed the formation of DNA DSB, probably resulting from replication fork collapse at damaged DNA sites (Fröls *et al.* 2007). These observations inspired us to investigate in this study if the cellular aggregation is causally linked to the presence of DSB in the genome. Indeed, the DSB-inducing agents bleomycin and mitomycin C caused the same phenotype of cellular aggregation as UV light. Similarly, the proliferation of the *S. shibatae* virus 1 (SSV1) can be induced by mitomycin C as well as UV light (Martin *et al.* 1984) indicating that the same internal signal cascades are involved.

However, it is still unclear how DSB DNA might be sensed in the cells and how the signal is further transferred to induce the cellular aggregation and DNA-repair reactions. A phototaxis mechanism is reported for *H. salinarum* that regulates the motor switch of the flagella. The UV light is sensed by the sensory rhodopsin (Htr) and activates a Che-like two-component system (Nutsch *et al.* 2003). However, neither of these components is known in *Sulfolobales*. In *Synechocystis* PCC6803 Che-like histidine kinases control the cell orientation to the light and type IV pilus biosynthesis (Bhaya *et al.* 2001).

We observed that strain Δ*sso0120* was more sensitive than wild type to DSB-inducing agents, suggesting that cell aggregation is required for efficient DNA repair. Although aggregation could simply reduce the exposure of cells to UV light by shading, we believe that aggregation might play a role in mediating the formation of mating pairs that allow DNA repair via homologous recombination among the partners.

Significantly enhanced exchanges of chromosomal markers upon treatment with UV light have been described for *Sulfolobus* (Wood *et al.* 1997; S. Fröls and C. Schleper, unpublished).

Furthermore, we found a slight, but significant upregulation of the *mre11* operon upon UV treatment in *S. solfataricus* using whole-genome microarrays (Fröls *et al.* 2007). This operon encodes homologues of the eukaryotic system involved in the DSB repair via homologous recombination (Hopfner *et al.* 2002; Constantinesco *et al.* 2004).

By integrating our observations and those cited above we think that recombinational repair via homologous recombination and DNA exchange via cell–cell contacts might be an important strategy to overcome DNA damage in *Sulfolobus* caused by UV light. Future studies will aim at investigating if this is indeed the case. It will also be interesting to elucidate the transcriptional regulation of the UV-induced genes, with the perspective to clarify the signal transduction pathways that sense UV irradiation or DNA damage in crenarchaeota.

## EXPERIMENTAL PROCEDURES

### Strains and growth

*Sulfolobus solfataricus* P1 (DSM1616), PH1 (Schleper *et al.* 1994), PH1-M16 (Martusewitsch *et al.* 2000) and PBL2025 (Schelert *et al.* 2004) and derived deletion mutants were grown aerobically at 80°C in the medium described by Brock *et al.* (1972), adjusted to pH 3 with sulphuric acid and supplemented with 0.1% (w/v) of trypton and 0.2% (w/v) of arabinose under moderate agitation (150 r.p.m. in a New Brunswick shaker). Growth of cells was monitored by measuring the optical density at 600 nm. Solid media were prepared by adding gelrite to a final concentration of 0.6% and $MgCl_2$ and $CaCl_2$ to 0.3 and 0.1 M respectively. Plates were incubated for 5 days at 78°C. For the propagation of plasmids *E. coli* strain DH5α was used. For the virus containing plasmids ElectroMAX™ *E. coli* Stbl4™ cells (Invitrogen, Germany) were used.

### UV light exposure

UV treatment of cells was performed under red dimmed light. Aliquots of 10 ml of *S. solfataricus* culture ($OD_{600}$ 0.3–0.5) were transferred to a 110 mm plastic Petri dish

and treated with a defined UV dose (λ 254 nm, UV-Stratalinker 1800, Stratagene). Treated cell suspensions were combined to a final volume of 20 ml. The mock-treated cultures were set for 5 s under red dimmed light. Treated cells were stored in the dark at room temperature for 15 min and were re-incubated at 78°C and 150 r.p.m. Samples taken at different time points were used for microscopy, cell vitality and electron microscopy.



**Electron microscopy and single-particle analysis**

For image processing, cells with attached pili were negatively stained with 2% uranyl acetate on glow-discharged carbon-coated copper grids. Electron microscopy was performed on a Philips CM120 electron microscope operating at 120 kV with a LaB6 filament. Images were recorded with a 4000 SP 4K slow-scan CCD camera at 60 000 × magnification at a pixel size of 5.0 Å at the specimen level with 'GRACE' software (Oostergetel *et al.* 1998). Single-particle analysis was performed with the Groningen Image Processing ('GRIP') software package on a PC cluster. Non-overlapping pili segments were extracted from the micrographs and aligned with correlation methods. The aligned projections were treated with multivariate statistical analysis in combination with hierarchical classification before final averaging (van Heel *et al.* 1992; Penczek *et al.* 1992).

**Plasmid construction for expression in *S. solfataricus* and *E. coli***

The genes *sso0117* and *sso0118* were cloned using primers 118-forward-NcoI and 117-reverse-BamHI in the same arrangement as found in the genomic context into the virus-based expression vector pMJ05 (Jonuscheit *et al.* 2003) via the entry vector pMZ1 (Zolghadr *et al.* 2007) yielding pSVA96. To express the whole *ups*-operon genes *sso0121-117* were cloned using primers 121-forward-NcoI and 117-reverse-BamHI via pMZ1 into pMJ05 resulting in pSVA125. To construct the expression plasmid for the signal peptide cleavage assay, *sso0118* was amplified using 118-forward-NcoI and 118-reverse-BamHI and cloned into pZA7, which added a HA-tag to the C-terminus of the protein and resulted in pSVA133. By NcoI–HindIII restriction the sso0118-HA part was then transferred into pBAD/Myc-HisA yielding pSVA134. To achieve coexpression with the peptidase a fragment containing *pibD* under the control of the T7 promoter was cloned from pUC18-pibD into pSVA134 by SphI restriction resulting in pSVA135. All plasmid vectors used in this study are listed in Table 3.

For the expression of SSO0117/0118 and the *ups*-operon in the Δ*flaJ* strain, cells were grown to an $OD_{600}$ of 0.2, and transformed with pSVA96, pSVA125 and pSVA31 as a control as described by Jonuscheit *et al.* (2003). After 2 days, cultures were transferred to medium containing 0.4% arabinose to induce the expression of the desired genes. After one transfer, cells were analysed at an $OD_{600}$ of 0.7 cells by electron microscopy.

**Table 3.** Plasmids used in this study.

| Plasmid name | | References |
|---|---|---|
| pET2268 | Vector containing *lacS* cassette | Szabó *et al.* 2007b |
| pZA7 | Transfer vector to add HA-tag to proteins | Szabó *et al.* 2006 |
| pUC18-*pibD* | pUC18 containing *pibD* under control of a T7 promoter | Szabó *et al.* 2007b |
| pMZ1 | Entry vector for virus vector | Zolghadr *et al.* 2007 |
| pMJ05[a] | Virus-based shuttle vector for *S. acidocaldarius* | Jonuscheit *et al.* 2003 |
| pSVA31 | pMJ05 containing *ABCE1* under control of a *araS* promoter | Albers *et al.* 2006 |
| pSVA37 | pET2268 containing up- and downstream flanking regions of *sso0120* | This study |
| pSVA96 | pMJ05 containing *sso0117/0118* under control of a *araS* promoter | This study |
| pSVA99 | pMJ05 containing *sso0120* under control of a *araS* promoter | This study |
| pSVA125 | pMJ05 containing *sso0121-117* under control of a *araS* promoter | This study |
| pSVA133 | pZA7 containing *sso0118* | This study |
| pSVA134 | pBAD/*Myc*-HisA containing *sso0118*-HA | This study |
| pSVA135 | pSVA134 containing *pibD* | This study |

a. Plasmids labelled bold are shuttle vectors for *S. acidocaldarius*/*E. coli*

**Construction of plasmids for the directed deletion of genes**

The up- and downstream flanking regions of *sso0120* were amplified using primer pairs KO-UP forward/KO-UP reverse and KO-DOWN forward/KO-DOWN reverse respectively (Table S1). The resulting fragments were cloned using KpnI/NcoI for the upstream flanking region (1099 bp) or BamHI/NotI for the downstream flanking region (924 bp) in pET2268, a vector containing the *lacS* cassette for selection, yielding pSVA37. Electroporation of the knockout plasmids and selection for correct deletion mutants were performed as described (Albers and Driessen 2007).

**Southern blotting**

Genomic DNA (8 ng) was digested with the appropriate enzymes and separated on 0.8% agarose gel. The gel was equilibrated in 20 × SSC and the DNA was transferred overnight to a positively charged nylon membrane (BIO-RAD, the Netherlands). DNA hybridization was performed in standard hybridization buffer. PCR products of both *lacS* and the *sso0120* gene were digoxigenin-labelled with the HighPrime Kit (Roche, the Netherlands). Detection was performed with a Lumi Imager (Roche, the Netherlands).

**Gene expression analysis**

Total RNA isolation and cDNA synthesis were performed as described previously (Zolghadr *et al.* 2007). Gene-specific primer sets (1–7, Table S1) were used to detect the presence of the genes in the *ups*-operon. PCR products were analysed on 0.8% agarose gels.

**Isolation of *E. coli* crude membranes**

BL21(DE3) (pLysS) *E. coli* cells were transformed with plasmids pSW017, pSW018, pSW019 and pSW020. The signal sequence cleavage assay was performed as described before (Szabó *et al.* 2007b). At an $OD_{600}$ of 0.6, the expression of the precursor genes, *sso0117/0118*, was induced by addition of 0.2% l-arabinose for 2 h. Subsequently, the expression of *pibD* was induced with 0.1 mM isopropyl-beta-d-thiogalactopyranoside (IPTG) for 2 h. The cultures were harvested, and cell pellets were re-suspended in 2 ml of buffer (50 mM TrisCl, pH 7.5, 1 mM EDTA). Crude membranes were isolated as described previously (Szabó *et al.* 2007b) and re-suspended in 50 mM TrisCl, pH 7.5. Cleavage of substrates was determined by SDS-PAGE and Western blot analysis of 5 µg of crude membranes using monoclonal anti-haemagglutinin (HA Tag) antibodies (Sigma).

**Microscopy and quantitative analysis of cell aggregate formation**

Cell aggregate microscopy was performed as described (Fröls *et al.* 2007). To quantify the formation of aggregates, the number of cells in aggregates and the number of aggregates were counted until 1000 or 500 single cells were observed. At least seven fields of views were analysed for each time point. To exclude that the cellular aggregates were not artefacts of the microscopic slide preparation only fields of views were analysed where the cells showed an even spreading. For statistic analysis the percentage of cells in aggregates ≥ 3 cells (to exclude the dividing pairs of cells) against the total amount of cells was calculated. Additionally the percentage of each aggregates size (from 3 to 15 cells) against the total amount of cells was determined to observe the time- and dose-dependent formation of cellular aggregates in a higher resolution.

**Analysis of the cell viability**

To analyse the cell viability the LIVE DEAD Baclight (Invitrogen) assay was used according to the manufacturer's instructions. Alternatively, a combined 4′,6-diamidino-2-phenylindole (DAPI) propidium iodide stain was used. At 6 h after UV treatment, samples of 20 µl of liquid cultures were mixed with 2 µl of propidium iodide (1:30 dilution in 10 mM TrisCl pH 7.5) and incubated for 15 min in the dark at room temperature. Microscopic slides were coated with 1% agar (10 mM TrisCl, pH 7.5) containing 0.2 µg $ml^{-1}$ DAPI. Propidium iodide-stained

culture (5 µl) was spread on the coated slide and immediately examined. To analyse the number of dead cells in aggregates in relation to the living cells, a minimum of 50 cellular aggregates of ≥ 3 cells were counted for each UV dose.





**Testing of various stress factors**

For the temperature shift, exponentially grown *S. solfataricus* cultures ($OD_{600}$ of 0.3–0.5) were transferred from 78°C to 88°C or to 65°C. Additionally control cultures were transferred to 78°C with gentle shaking at 150 r.p.m., and at time points up to 10 h after transfer, samples were analysed for aggregation.

For the pH shift experiments, exponential-grown *S. solfataricus* cells were harvested and re-suspended in an identical volume of Brock's basal salt medium supplemented with d-arabinose (0.2%) and tryptone (0.1%) at pH values of 2.5, 3 and 4 respectively. Growth was continued at 78°C. Samples for the quantitative analysis of the cellular aggregation were taken up to 8 h after the pH shift. For the treatment with the DSB-inducing antibiotics, an exponential *S. solfataricus* culture was treated with 3 µg $ml^{-1}$ bleomycin (Bleocin, Calbiochem) or 5, 10 and 15 µg $ml^{-1}$ mitomycin C (Sigma). Growth was continued at 78°C and cell samples taken up to 10 h after antibiotics additions were plated on Brock's solid media. Survival rates confirmed the use of a non-lethal drug concentration for both antibiotics as described (Cannio *et al.* 1998; Reilly and Grogan 2002; Kosa *et al.* 2004).

## ACKNOWLEDGEMENTS

The work has been funded by Deutsche Forschungsgemeinschaft within SPP1112 and by the University of Bergen. D.T. is funded by the Marie Curie research training network MRTN-CT-2006-033499. S.-V.A. and B.Z. were supported by a VIDI grant from the Dutch Science Organization (NWO). M.A. was funded by an Ubbo-Emmius scholarship of the University of Groningen.

# 3

# UV-INDUCIBLE DNA EXCHANGE IN HYPERTHERMOPHILIC ARCHAEA MEDIATED BY TYPE IV PILI

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*Molecular Microbiology (2011) 82, 807-817*

## ABSTRACT

Archaea, like bacteria and eukaryotes contain proteins involved in various mechanisms of DNA repair, highlighting the importance of these processes for all forms of life. Species of the order Sulfolobales of hyperthermophilic crenarchaeota are equipped with a strongly UV-inducible type IV pilus system that promotes cellular aggregation. Here we demonstrate by fluorescence *in situ* hybridization that cellular aggregates are formed based on a species-specific recognition process and that UV-induced cellular aggregation mediates chromosomal marker exchange with high frequency. Recombination rates exceeded those of uninduced cultures by up to three orders of magnitude. Knockout strains of *S. acidocaldarius* incapable of pilus production could not self-aggregate, but were partners in mating experiments with wild-type strains indicating that one cellular partner can mediate the DNA transfer. Since pilus knockout strains showed decreased survival upon UV treatment, we conclude that the UV-inducible DNA transfer process and subsequent homologous recombination represents an important mechanism to maintain chromosome integrity in *Sulfolobus*. It might also contribute substantially to the frequent chromosomal DNA exchange and horizontal gene transfer in these archaea in their natural habitat.

## INTRODUCTION

The damaging effect of UV irradiation on the integrity of DNA is well known and the understanding of the repair mechanisms in organisms that are exposed to sunlight is of fundamental interest, in particular since the depletion of the Earth's ozone layer increases the deleterious effect of solar radiation (McKenzie *et al.* 2007). Bacteria, Archaea and Eukaryotes all have large numbers of proteins that are involved in DNA repair pathways of different types, reflecting the importance of these mechanisms for all life forms (White 2003). Some archaea have photolyases that directly repair DNA photoproducts, such as cyclobutyl pyrimidine dimmers (CPD). All seem to contain nucleotide excision repair systems that allow repair of UV-induced DNA lesions and also lesion bypass polymerases are found widespread, that bypass unrepaired damage during replication (Boudsocq *et al.* 2001). The information processing machineries of archaea are generally most closely related to eukaryotes and also the DNA repair systems are most similar to the eukaryotic counterparts or only occur in the eukaryotic/archaeal lineages, such as the XPF proteins of the nucleotide excision repair (White 2003). The proteins RadA, Mre11, Rad50, and the archaea-specific nuclease NurA and helicase HerA are most probably involved in DNA end repair (Delmas *et al.* 2009; Quaiser *et al.* 2008).

In a recent genome-wide microarray study of the UV response, we and others have shown that the hyperthermophilic archaeon *Sulfolobus* does not express an inducible set of proteins known to be involved in DNA repair, which would be comparable to an SOS response in bacteria. Instead the most strongly transcriptionally induced genes were of unknown function or encoded functions that are not normally associated with a UV-specific response (Fröls *et al.* 2007; Götz *et al.* 2007). Among the most strongly induced genes was the *ups*-operon (UV-induced pili of *Sulfolobus*) encoding a type IV pili assembly system, which consists of a secretion ATPase UpsE, an integral transmembrane protein UpsF, two pilin-like proteins UpsA and B and a protein of unknown function UpsX (Fröls *et al.* 2008). The *ups*-operon was shown to assemble pili on the cell surface of *S. solfataricus* which led to pili-mediated cell aggregation after UV stress (Fröls *et al.* 2008). Type IV pili represent multifunctional filaments that are involved in bacterial twitching and gliding motility, surface and cell to cell adhesion, immune escape, formation of biofilm, secretion, phage and signal transduction and DNA uptake in various bacteria, as opposed to the type IV secretion systems that are involved in conjugation processes (Filloux 2010). All sequenced Sulfolobales genomes possess the *ups*-operon.

In addition we have demonstrated that DNA double strand breaks occurred in the cells between 2 and 6 hours after UV treatment, but not directly after the irradiation, indicating that they were a product of cellular repair or of replication processes

rather than of direct damage (Fröls *et al.* 2007). Similar observations of DNA double strand breaks have been made decades ago in *Escherichia coli* (Bonura and Smith 1975) and more recently also in human skin cells (Garinis *et al.* 2005), indicating that their formation is a common but indirect and somewhat underexplored consequence of UV irradiation that could favour homologous recombination in the cells.

Here we demonstrate that the pili-dependent UV-induced cellular aggregation leads to efficient exchange of chromosomal markers among *Sulfolobus* cells of the same, but not of different species. The knockout of pili abolished DNA exchange and led to decreased survival upon UV exposure, indicating that intra-species DNA exchange increases fitness of the cells. DNA repair based on homologous recombination might be active potentially at regions with DNA double strand breaks formed after failed attempts to repair DNA by direct repair mechanisms.



## RESULTS

### UV-induced pilus formation and cellular aggregation in Sulfolobales

In order to explore and compare the extent of pili formation in different species of Sulfolobales, cells of *S. acidocaldarius*, *S. tokodaii* and *S. solfataricus* (Table 3) were exposed to UV-C light (dose 50 J/m$^2$ ) and were inspected for pili formation by electron microscopy. Pili were found abundantly on all strains after UV exposure (Figure 1), but not on untreated cells. The length of the *S. tokodaii* pili appeared with max 0.2 µm comparable to the *S. solfataricus* pili (length 0.2-0.3 µm) (Fröls *et al.* 2008), whereas the UV-induced pili of *S. acidocaldarius* were only up to 0.1 µm in length, but more abundant. Pili were spread over the whole cell surface sometimes as small bundles, but were not polarized at one side of the cell. The differences in pilus morphology of the three strains might be due to the differences in the

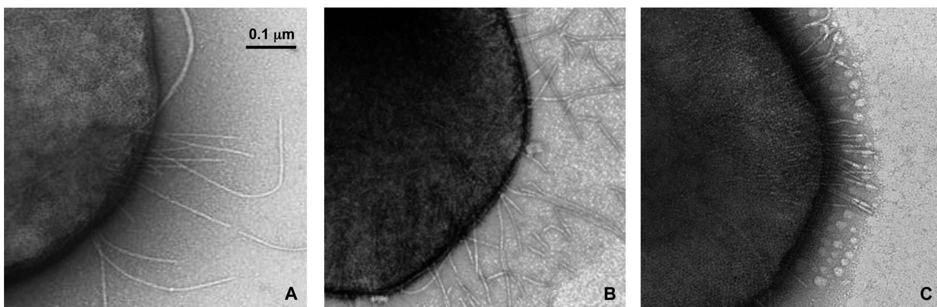


**Figure 1.** UV-inducible pili on electron micrographs of *S. solfataricus* (A), *S. tokodaii* (B) and *S. acidocaldarius* (C). Pili exhibit similar diameters, but differences in length between the strains, with *S. acidocaldarius* producing the shortest but also most abundant pili.

amino acid sequence of the pilins (between 61 and 66% amino acid similarity, see Figure S1) or due to different levels of their expression.

The three strains produced large amounts of cellular aggregates upon UV irradiation. These were particularly large in S. *acidocaldarius* with up to approx. 50 cells or more per aggregate (Figure 2). One hour after UV irradiation with a dose of 50 J/m$^2$ already 30% of the cells were found in aggregates and later more than 90% (Figure S2). These numbers are rather underestimates, because the amount of cells in large aggregates was difficult to count.

The process of aggregation was time dependent with a maximum of cellular aggregation observed from three to six hours after UV treatment, when nearly all cells were found in aggregates. Although a basic level of around 10-20 cells in aggregates per 100 free cells were found in non-irradiated control cultures, those were considerably smaller than UV-induced aggregates (Figure S2). Deaggregation started from 8-10 hours after UV treatment and no aggregates were found any more 24 hours after UV treatment. A similar trend had been observed with UV-induced aggregations in S. *solfataricus* (Fröls *et al.* 2007). Aggregate formation depended on the UV dose, with the largest number of aggregates occurring between 25 and 75 J/m$^2$ (Figure S3). As demonstrated by a LiveDead stain with fluorescent dyes, at least 80% of the cells in aggregates of *S. acidocaldarius* exposed to a UV dose of 50 J/m$^2$ were viable, i.e. had an intact plasma membrane (Figure S4) indicating that the process of aggregation was not caused by accumulation of UV-destroyed and dead cells but reflected a specific response to UV stress as in *S. solfataricus* (Fröls *et al.* 2008). Aggregation was also inducible by the DNA strand-break inducing agent bleomycin at non-lethal concentrations (Figure S5). Aggregates appeared first 3 hours after DNA-damaging treatment with 30 µg/ml bleomycin, with a maximum number of aggregates after 6 to 8 hours. Correspondingly, pili were observed in electron microscopy on the cells treated with bleomycin. The DSB-inducing agents bleomycin and mitomycin C had been observed to also induce cellular aggregation of *S. solfataricus* strains P1, PH1-M16 and PBL2025, but not of the knockout strain of the ATPase UpsE (Table 3) (Fröls *et al.* 2008).

**Species-specific aggregation**

In order to explore the specificity of cell-cell recognition among UV-induced *Sulfolobus* cells, we have designed species-specific oligonucleotides targeting the small subunit ribosomal RNA in fluorescent *in situ* hybridizations (FISH). The probes had two to three mismatches each to the two other related species (Table S1).

Their specificity was confirmed by FISH analyses on pure cultures that were competitively challenged with the different probes (not shown). After UV irradiation and mixing of cells from two different species cellular aggregation was visualized with the probes (Figure 2). Large, homologous cell clusters were formed

as indicated by the homogenously coloured green and red aggregates, respectively, as shown for *S. solfataricus* and *S. tokodaii* in Figure 2A and for S. *acidocaldarius* and *S. tokodaii* in Figure 2B. This indicated that cellular aggregation was based on a species-specific recognition process. Respective control experiments revealed a homogenous distribution of single cells and only few aggregates, demonstrating that the aggregate formation was indeed UV-induced and not a biased result of the FISH procedure (Fig 2A and 2B, lower control panels). Only few, very large aggregates were occasionally observed that contained differentially stained cells. However, even in those large aggregates, no completely random mixing of colours

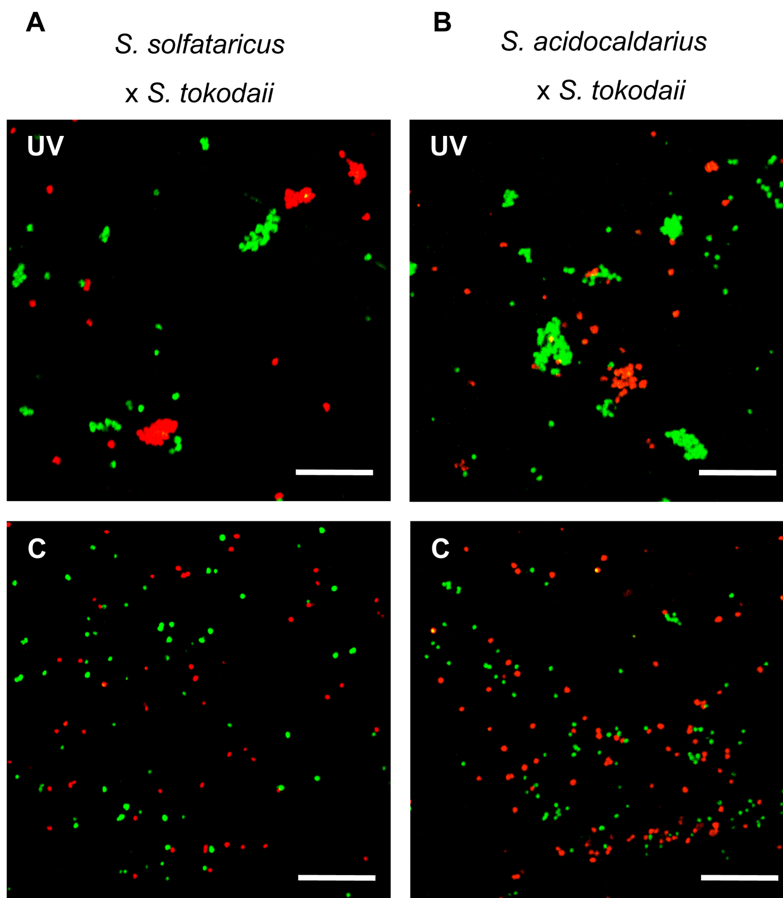


**Figure 2.** Fluorescent *in situ* hybridization (FISH) with species-specific probes. UV-irradiated mixture of cells (UV) and control cultures (C) of *S. solfataricus* (green) with *S. tokodaii* (red) (A) and S. *acidocaldarius* (green) with *S. tokodaii* (red) (B). When cells were mixed, mostly homologous aggregates were formed, indicating a highly species-specific recognition mechanism (UV). Scale bar corresponds to 10 µm.

was seen, but rather batches of cells of the same colour seemed to aggregate with batches of the other colour to form a huge aggregate. Quantitative analysis of the hybridized homogenous and heterogeneous aggregates confirmed that only a minor fraction of all formed aggregates consisted of two strains, i.e. *S. solfataricus* mixed with *S. tokodaii* (12.8±6.9%) and *S. acidocaldarius* mixed with *S. tokodaii* (10.3±4.5%). From the few aggregates seen in control experiments without UV irradiation a significantly higher proportion consisted of the two mixed strains with UV treatment (*S. solfataricus* x *S. tokodaii*: 40.6±1.7%; *S. acidocaldarius* x *S. tokodaii*: 28.5±2.5%). These data further demonstrate that the process of UV-induced aggregation is species-specific.



**UV-inducible pili mediate increased exchange of chromosomal markers among *Sulfolobus* cells**

The formation of aggregates in UV-treated cultures of *Sulfolobus* led to the hypothesis, that these are used for DNA exchange assisting DNA damage repair by homologous recombination. In order to test this hypothesis, mating experiments with a *lacS*/*pyrEF* double mutant of *S. solfataricus* (Table 3) defective in ß-galactosidase and *de novo* uracil synthesis, respectively (Martusewitsch *et al.* 2000) was mixed with wild-type cells of strain P1. The mating assay was based on the transfer of the ß-galactosidase encoding reporter gene *lacS* from the wild-type strain P1 to the *lacS*-deficient strain PH1-M16. Recombinants (ex-conjugants) were screened for, which were 5-FOA resistant (thus $pyrEF^{-}$) and formed blue colonies when incubated with X-gal, thus $lacS^{+}$. The recombination frequencies were up to three orders of magnitude higher than spontaneous mutations of the wild-type strain alone that resulted in the same phenotype (Table 1). No reversion events of the mutated *pyrEF* or *lacS* genes of the PH1-M16 ($lacS^{-}/pyrEF^{-}$) strain were observed with or without UV irradiation. The $lacS^{+}/pyrEF^{-}$ genotype of recombinants was verified by Southern Blot analyses (Figure 3). No recombination events were observed without UV irradiation.

To better characterize the role of pili in DNA transfer the genes for the central ATPase UpsE and an integral transmembrane protein UpsF from the *ups*-operon were removed via a marker-less exchange method in *S. acidocaldarius* (Wagner *et al.* 2009). In this species the genetic toolbox is best developed and the knockout of *upsEF* genes is one of the first mutants that has been prepared via a single crossover (Wagner *et al.* 2009). Southern blotting confirmed the deletion in the mutant (Figure 4). As expected we could not observe any pili in the deletion strain before and after UV irradiation, and no significant increase of aggregate formation (Figure S2), confirming earlier results with *S. solfataricus* that the pili are necessary for aggregate formation (Fröls *et al.* 2007).

We then used a series of *ups*/*pyrE* double mutants in mating experiments (Table 3). The assays detected transfer of chromosomal DNA from either parental

**Table 1.** Recombination frequencies in *S. solfataricus* upon UV-treatment with 75 J/m$^2$ UV (A, B) or 50 J/m$^2$ (C, D).

| Experiment | | Recombination mix cfu/ml (*lacS*$^-$/*pyrEF*$^-$) x (*lacS*$^+$/*pyrEF*$^+$) | Recombination frequencies[a] (*lacS*$^+$/*pyrEF*$^-$) | Mutation frequencies[b] (*lacS*$^+$/*pyrEF*$^-$) |
|---|---|---|---|---|
| A | 75 J/m$^{-2}$ | 3.80 x $10^7$ | 1.11 x $10^{-2}$ | 1.90 x $10^{-5}$ |
| | Control | 1.87 x $10^8$ | -[c] | 1.59 x $10^{-5}$ |
| B | 75 J/m$^{-2}$ | 2.77 x $10^7$ | 4.85 x $10^{-3}$ | 3.21 x $10^{-6}$ |
| | Control | 2.10 x $10^8$ | - | 1.00 x $10^{-4}$ |
| C | 50 J/m$^{-2}$ | 3.67 x $10^7$ | 1.09 x $10^{-3}$ | n.d.[d] |
| | Control | 8.80 x $10^7$ | - | n.d.[d] |
| D | 50 J/m$^{-2}$ | 1.42 x $10^8$ | 3.11 x $10^{-3}$ | 2.14 x $10^{-5}$ |
| | Control | 2.39 x $10^8$ | - | 2.15 x $10^{-5}$ |

[a] Recombination frequencies were determined as event per cell; the median of the positive recombinants (*lacS*$^+$/*pyrEF*$^-$) cfu/ml with selection (5-FOA) were determined and divided by the median of the cfu/ml from all observed colonies under non-selective conditions.
[b] Mutation frequencies for strain P1 as event per cell.
[c] No recombination events observed.
[d] Not determined.

strain to the other, leading to recombination events that restored the function of the biosynthetic gene (*pyrE*) inactivated by a different mutation in the two strains (Dudás and Chovanec 2004). To provide multiple options for this assay, two corresponding pairs of *pyrE* strains were constructed. Each pair included one mutant with a short deletion centered on *pyrE* nt 163, and one with a point mutation at *pyrE* nt 335 (see Methods and Table S2). The two pairs of strains differed with respect to their *upsEF* genotype; one pair (MR31 and JDS28) was *ups*$^+$ (i.e., Pil$^+$ ) the other pair (SA1 and DG253) was *ΔupsEF* (i.e., Pil$^-$). Despite the fact that the *pyrE* marker spacing was the same in both crosses, MR31 mated with JDS28 yielded 20-30 Pyr$^+$ recombinants, whereas SA1 with DG253 yielded none (Figure 5). This indicated that the type IV pili made by the ups-system are necessary for DNA transfer even without UV treatment in *S. acidocaldarius.* We also evaluated "unequal" mating in which one partner was Pil$^+$ and the other Pil$^-$. These matings yielded recombinants (Figure 5), indicating that successful DNA exchange requires only one of the two parental strains to have pili.

We then evaluated "unequal" (i.e., Pil$^+$ and Pil$^-$) combinations in which only one of the two parental strains was irradiated before mating. To provide multiple comparisons, members of isogenic Pil$^+$ and Pil$^-$ pairs (JDS28/DG253, or MR31/SA1) were each mated to a common Pil$^+$ *pyrE* mutant, JDS183. As shown in Figure 5, when one of the strains was UV-treated, an increase in yield of recombinants was

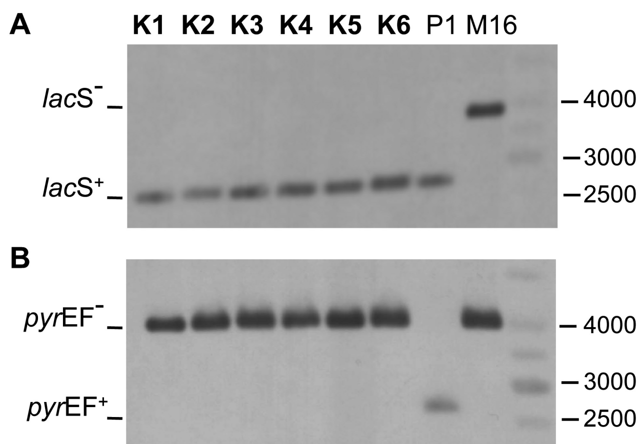


**Figure 3.** Southern-blot analysis confirming the resulting (*lacS*$^+$/*pyrEF*$^-$) genotype of isolated positive recombinants (K1-K6), after mating of *S. solfataricus* P1 (*lacS*$^+$/*pyrEF*$^+$ ) with *S. solfataricus* M16 (*lacS*$^-$/*pyrEF*$^-$) using *lacS*-specific DNA probes (A) and *pyrEF*-specific DNA probes (B).

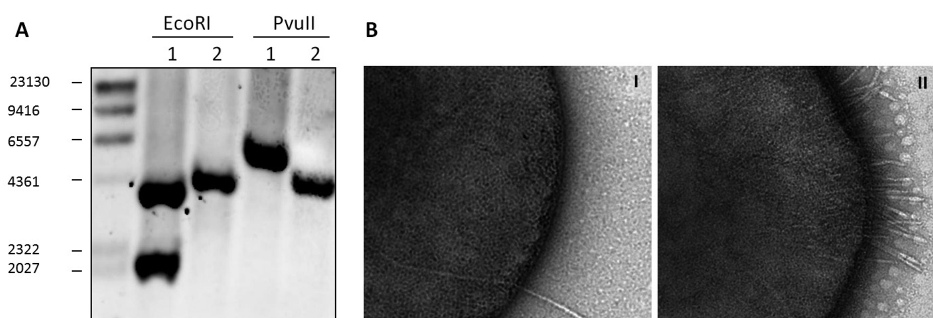


**Figure 4.** Southern blot analysis of wild-type Pil$^+$ *S. acidocaldarius* MR31 (1) and Pil$^-$ (2) strain (A) to confirm knockout of *upsEF* genes. Expected fragment sizes for WT MR31: 3906 bp and 2128 bp for EcoRI and 6785 bp for PvuII (1). Expected fragment sizes for knockout of *upsEF*: 4593 bp for EcoRI and 5344 for PvuII (2). Electron micrographs of Pil$^-$ strain with UV (BI) and Pil$^+$ strain with UV (BII). Only flagella like structures can be observed on the cell surface of UV-treated Pil$^-$ cells and no short straight UV pili (B). Scale bar is 0.2 µm in length.

only observed when the UV-irradiated strain was Pil$^+$. UV treatment of only the Pil$^-$ strain never elevated the recombination frequency (Figure 5, Table S3). Finally, for assays in which a Pil$^+$ and a Pil$^-$ strain had been mated, the *upsEF* genotype of recombinants was scored by PCR analyses (Table 2). Between 62 and 81% of the *pyrE*$^+$ colonies were *upsE*$^+$, meaning that at least two-thirds of the Pyr$^+$ recombinants were pili producers.

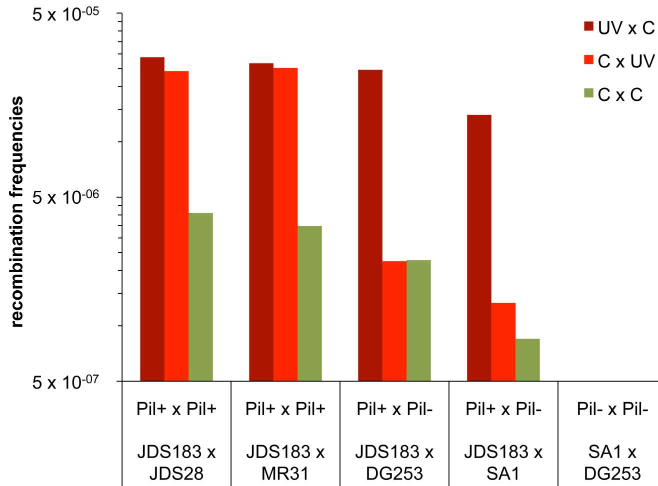


**Figure 5.** Recombination frequencies of mating experiments with *S. acidocaldarius* wild-type and delta-pili mutants. Two different strains treated with (UV) or without (C) UV, were mixed together in different combinations and plated on selective media. All strains contained mutations in the *pyrE* gene (involved in *de novo* uracil biosynthesis) located at different positions, such that recombination between two strains can restore the wild-type phenotype. Bars represent the median of 3 or 4 independent mating experiments each.

**Table 2.** Genotype analysis[a] of recombinants of *S. acidocaldarius* with and without prior UV treatment.

| Mixed mating strains | | No. of tested recombinants | *upsE* genotype of recombinants[a] | | |
|---|---|---|---|---|---|
| | | | pil⁻ (*upsE*⁻) | pil⁺ (*upsE*⁺) | %pil⁺ (*upsE*⁺) |
| WT pili x Δ pili | UV x C | 16 | 3 | 13 | 81.25 |
| (JDS183 x SA1) | C x UV | 16 | 4 | 12 | 75 |
| | C x C | 16 | 4 | 12 | 75 |
| WT pili x Δ pili | UV x C | 15 | 5 | 10 | 66.7 |
| (JDS183 x DG253) | C x UV | 16 | 6 | 10 | 62.5 |
| | C x C | 16 | 3 | 13 | 81.25 |

[a] Analysis performed by PCR with primers flanking the *upsE* locus.

Attempts to demonstrate marker recombination with lysed cells from one partner and UV-irradiated intact cells of a second partner failed in all cases, indicating that the DNA transfer does not involve the uptake of free DNA, as was also shown before (Grogan 1996).

### Increased fitness of Pil⁺ strains upon UV treatment

In order to analyze the survival of Pil⁺ and Pil⁻ strains upon UV treatment, the four different strains of *S. acidocaldarius* were exposed to different doses of UV light and subsequently plated (Figure 6). A significant reduction in plating efficiency was observed for the pili knockout strains compared to Pil⁺ strains, indicating that the





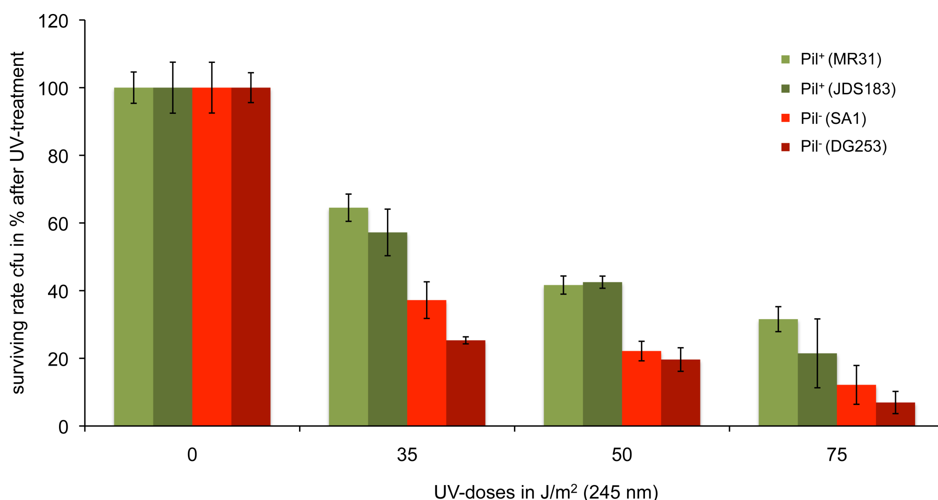


**Figure 6.** Survival frequencies of *S. acidocaldarius* wild-type (green) and delta pili strains deficient in pili formation (red) treated with different UV doses. Results are based on 3 independent experiments.

**Table 3.** Strains used in this study.

| Strain | Genotype or description | Source or reference |
|---|---|---|
| *S. acidocaldarius* MR31 | Deletion of 18 bp in *pyrE* (nt 154-171) | Reilly and Grogan (2001) |
| *S. acidocaldarius* JDS28 | Point mutation in *pyrE* (A > T at nt 335) | Sakofsky *et al.* (2011) |
| *S. acidocaldarius* JDS183 | Frameshift mutation in *pyrE* (duplicated T at nt 44) | Grogan and Hansen (2003) |
| *S. acidocaldarius* SA1 | Δ*upsEF* in MR31 | D.W. Grogan |
| *S. acidocaldarius* DG253 | Δ*upsEF*, point mutation in *pyrE* (A > G at nt 335) | D.W. Grogan |
| *S. solfataricus* P1(DSM1616) | Wild type | DSMZ |
| *S. solfataricus* PBL2025 | *S. solfataricus* 98/2 Δ(*sso3004-3050*) | Schelert *et al.* (2004) |
| *S. solfataricus* Δ*upsE* | *S. solfataricus* PBL2025 Δ*sso0120* | Fröls *et al.* (2008) |
| *S. solfataricus* PH1-M16 | *S. solfataricus* P1 Δ*lacS*, Δ*pyrEF* | Martusewitsch *et al.* (2000) |
| *S. tokodaii* (DSM16993) | Wild type | DSMZ |

pilus-mediated process considerably increases the fitness of cells. The effect was particularly obvious at relatively low doses of UV light, i.e. 35 and 50 $J/m^2$, where approx. 60 and 40% of the wild-type cells survived, but only around 30 and 20%, respectively, of the mutant strains (Figure 6). The significance of these results was confirmed by statistic evaluation of the yielded survival rates (see Table S4). First a two-way ANOVA was performed to revise variance among the strains and the used UV doses. The calculation revealed highly significant differences (p-value $< 0.001$) among the tested strains (p-value $9.1 \times 10^{-7}$) and the UV doses (p-value $2.2 \times 10^{-16}$). These results confirmed that the cellular resistance to UV damage is dependent on the existent of a functional *ups*-operon and mediated processes.

## DISCUSSION

Homologous recombination (HR) is a mechanism that is used in all domains of life for repair of double-strand DNA breaks (Dudás and Chovanec 2004) and restart stalled DNA replication forks. Furthermore, HR is considered an important mechanism to maintain genomic cohesion among individuals of a bacterial or archaeal species (Papke *et al.* 2007), but is also the prerequisite for exchange of accessory genomic functions, e.g., antibiotic resistance and pathogenicity islands (Deveau *et al.* 2010; Gudbergsdottir *et al.* 2011; Manica *et al.* 2011). While it is generally accepted that genetic exchange among microbes must occur at high frequencies, relatively little is known about the conditions which trigger effective gene exchange among individuals of a microbial community. As in bacteria, a number of mobile genetic elements, such as viruses, plasmids and insertion sequences have been described in archaea, all of which can promote directly or indirectly HR. Conjugative plasmids have so far only been described in the crenarchaeal order Sulfolobales (Schleper *et al.* 1995; She *et al.* 1998; Stedman *et al.* 2000). They seem to spread effectively among strains (Schleper *et al.* 1995). However, the mechanism of conjugation is not understood and only few genes on these plasmids show remote similarity to genes involved in conjugation of bacteria (She *et al.* 1998). In the archaeon *Haloferax volcanii* an effective exchange of chromosomal markers has been demonstrated (Rosenshine *et al.* 1989) that seems to involve a mutual exchange of genomic material via cytoplasmic bridges. Similarly, frequent exchange of chromosomal markers, independent of the presence of known conjugative elements in the cells, has been demonstrated in plating experiments with *Sulfolobus acidocaldarius* (Grogan 2009) which increases with UV treatment (Schmidt *et al.* 1999). Furthermore, molecular studies by Whitaker *et al.* (Whitaker *et al.* 2005) on 60 different strains of *Sulfolobus islandicus* demonstrated homologous recombination for all six loci investigated. Frequent recombination among naturally occurring *Ferroplasma acidarmanus* strains

resulting in a mosaic gene pool were described in a metagenomic study (Eppley *et al.* 2007). Furthermore, Papke *et al.* (Papke *et al.* 2004) reported on vigorous recombination among *Halorubrum* strains with an identical 16S rRNA gene in a solar saltern that resulted in a population approaching linkage equilibrium. While frequent exchange of chromosomal markers has been described the mechanisms and driving forces underlying lateral gene transfer (LGT) remain largely unknown in archaea, and also in bacteria. The UV-inducible chromosomal DNA exchange in *Sulfolobus* that we describe here might not only represent an effective means to repair DNA by homologous recombination, it might also promote frequent DNA exchange in natural communities. We have observed up to three orders of magnitude higher recombination frequencies after UV treatment.

Different from many bacterial conjugation or DNA exchange processes described so far, the DNA transfer in *Sulfolobus* (i) is strongly inducible by UV treatment, (ii) involves the transfer of chromosomal markers and (iii) is dependent on the formation of type IV pili by at least one partner. UV treatment of only one (pili-producing) partner was sufficient to increase the yield of DNA exchange. This increase only occurred when the Ups$^+$ partner was irradiated, whereas UV treatment of the partner without pili did not stimulate recombination. Furthermore, more than two-thirds of the *S. acidocaldarius* Pyr$^+$ recombinants that resulted from mating between an *ups*$^+$ and Δ*upsEF* strain were Pil$^+$. We thus conclude that the high level of transfer seen after UV irradiation involves an active recruitment of DNA by a UV-damaged cell that produces pili. The DNA donor can either be a non-damaged or a UV-damaged cell (see Figure 5). It has not been established, however why a significant minority of recombinants from these matings are Δ*upsEF*. Possibilities include non-selected transfer of the *upsEF* deletion to the (originally Ups$^+$) recipient, or reverse transfer of DNA between paired cells that brings *pyrE* DNA into the Δ*upsE* partner. It might, however, also be possible that the transfer is to some extent bidirectional with one partner being UV-activated and that our ex-recombinants are in part the results of mutual transfer of the two markers.

Interestingly, type IV pili of bacteria are usually not associated with conjugation or with cell-to-cell transfer of DNA but are rather involved in DNA uptake from the environment (Chen *et al.* 2005) or in other processes, such as motility (Ayers *et al.* 2010). However, in two cases type IV pili have recently been identified to be involved in cellular DNA transfer in bacteria (Carter *et al.* 2010; Kim and Komano 1997). Whereas the conjugative type IV pilus of R64 is plasmid encoded (Kim and Komano 1997), a type IV pilus involved in conjugation is encoded in the pathogenicity island PAPI-1 in *Pseudomonas* strains. Transmission of the island is dependent on a factor from the core genome, which probably restricts the range

of bacteria that can participate in the exchange of this genetic island (Carter *et al.* 2010). Compared to this, the *Sulfolobus* systems seems to be fully chromosomally derived: the Ups type IV pili are genome encoded, no GC skew is evident to the surrounding genomic sequences and their genetic context does not indicate that they are derived from a former conjugative plasmid. Furthermore, they do not only transfer a particular chromosomal island or plasmid, but rather transfer unspecific regions or perhaps even the whole genome during UV-induced DNA repair (Wood *et al.* 1997 and *S. solfataricus* assay in this study).

A variety of type IV pili have been described in archaea and their prepilins are processed by a dedicated type IV prepilin signal peptidase PibD/FlaK like the bacterial ones. The pili have been shown to be involved in surface motility, swimming, surface attachment and biofilm formation (Albers and Pohlschroder 2009; Koerdt *et al.* 2010). As the structural subunits of the ups-system in the different Sulfolobales strains are highly conserved, but the appearance of the pili strikingly different, we propose that the species specificity of aggregation depends on the recognition of the pilin subunits with the cell surface. Successful cell aggregation and subsequent DNA exchange will only take place, when the pili have determined the "host" cell being from the same species ensuring that DNA exchange is restricted to species from within the same gene pool. However, it could as well be possible that additional species-specific surface markers function in the recognition process.

In conclusion we describe a novel UV-inducible DNA transfer in hyperthermophilic Sulfolobales. It is mediated by type IV pili and loss of these pili reduces fitness of cells after UV damage, either by abolishing the DNA transfer process or by another as yet unknown mechanism. The DNA transfer triggers homologous recombination among cells in a population and might thus significantly promote horizontal gene transfer in naturally occurring communities. It remains to be shown if UV-induced DNA exchange mechanisms are found more widespread in other archaea and bacteria.

## EXPERIMENTAL PROCEDURES

### Growth conditions and UV light exposure

All *Sulfolobus* strains were grown at 78°C in Brock`s medium (Brock *et al.* 1972) or alternatively in a less complex mineral mixture (Grogan and Gunsalus 1993), adjusted to pH 3 with sulphuric acid and supplemented with 0.1% (w/v) of trypton or NZ-Amine ; 0.2% (w/v) of xylose and 10 µg/ml of uracil when necessary. Growth of cells was monitored by optical density measurements at 600 nm. For solid media the medium was supplemented with 1.5% gelrite. Plates were incubated for 5-6 days at 78°C. UV light exposure of *S. solfataricus* and *S. tokodaii*

was performed for a few seconds according to the desired UV dose as described in Fröls *et al.* (2008). For UV light exposure of *S. acidocaldarius* 25 ml of culture ($OD_{600}$ 0.2-0.4) were treated with a defined UV dose (254 nm, Spectroline, UV-crosslinker) in a plastic Petri dish and cells were immediately plated. Samples taken at different time points were analyzed in phase contrast microscopy, live and dead assay and electron microscopy. Optimal pili formation was found 3 hours after UV treatment for *S. acidocaldarius* and *S. solfataricus* and 6 hours for *S. tokodaii*.



**Electron microscopy analysis**

For image processing, cells were negatively stained with 2% uranyl acetate on carbon-coated copper grids. Transmission electron microscopy was performed on s Philips CM10 electron microscopy operating at 100kV.

**Light microscopy and quantitative analysis of aggregates**

Preparation of cells and light microscopic analysis was performed as described by Fröls *et al.* (2008). To quantify the number of aggregates, the cells were counted until at least 1000 single cells were observed. For the quantitative analysis the percentage of cells in aggregates (≥3 cells) against total amount of cells was calculated.

**Fluorescence *in situ* hybridization**

For fluorescence *in situ* hybridization (FISH) samples of single and mixed *Sulfolobus* strains, with and without UV treatment were used. For strain combination experiments, cultures were mixed in a stoichiometric ratio of 1:1, directly upon treatment. Flasks were stored in the dark for 15 min at room temperature and incubation was continued at 78°C for 6 hours with mild shaking at 150 rpm. After 6 h of incubation, samples were diluted 1:10 in Brock basal salt medium, 10 µl of culture were placed into a well on a FISH microscope slide (Marienfeld) and dried at RT. For fixation 10 µl of 37% formaldehyde was placed on top of each well and incubated for 20 min at RT. Afterwards formaldehyde was replaced by 10 µl of 1x PBS buffer and this was incubated for 2-3 min at RT to wash the cells. Fixed samples on the microscope slides were directly used for hybridisation or stored at -20°C. FISH was carried out as described in detail elsewhere (Daims *et al.* 2005). For probe sequences please see Table S1.

Following FISH, slides were embedded in Citifluor (Citifluor AF1; Citifluor Ltd., London UK) and examined using a confocal laser scanning microscope (LSM 510Meta; Carl Zeiss, Jena, Germany). For the quantification of the aggregates three technical replicates were used and hybridized in replicates, respectively (resulting in a total of six hybridizations per mixing experiment). After FISH, hybridizations were recorded using the tile scan function of the LSM510 Meta, allowing basically the recording of the whole microscopic well. Subsequently, aggregates were

counted manually following the above described criteria, with ≥3 cells counted as an aggregate. Furthermore, the aggregates were classified as species-specific (=aggregate with cells from only one species) or as mixed aggregate. In the latter case, an aggregate consisted of the two mixed strains, whereupon the mixing ratio was regarded as irrelevant; e.g. a mixture of five cells from one strain and a single cell of another strain was classified as mixed aggregate. In total more than 1000 aggregates were counted.

**Plasmid cloning for gene deletion and mutant construction**

The upstream and downstream flanking regions of *Saci_upsEF* were amplified using primer pairs 1-forward/11-reverse (GCGGGC-CGCGGGATATAGAAGTTGAAA GTGGAC/GCGCCCGGATCCCAGACAGAAAAATGAAAGTTATTCAAGG) and 12-forward/13-reverse (CGCGCCCGGGCAAGTCTTATATAGAATATACGGAG ACG/CGCGCTGCAGCATTTAGGAGAAGAGCGAC) respectively. PCR products were cloned using SacII/BamHI for the upstream flanking region and SmaI/ PstI digestion for the downstream region in Δ2pyrEF, a vector containing the *pyrEF* cassette for selection (Wagner *et al.* 2009), yielding pGA5. pGA5 was methylated as described before (Kurosawa and Grogan 2005) and transformed into MR31, integrants were then selected on plates lacking uracil. Subsequently they were streaked out on second selection plates containing 5-FOA to force looping out of the plasmid by homologous recombination. Resulting colonies were tested by PCR for successful deletion of the *upsEF* locus using primer pairs 16-forward/15-reverse (GGCATGGATCAGGTATTAGCAGAG / CCATAAGTAT GCAGTTATCAGCTCG) and 1-forward/13-reverse (GCGGGCCGCGGGATATA GAAGTTG-AAAGTGGAC / CGCGCTGCAGCATTTAGGAGAAGAGCGAC).

DNA sequencing on a PCR product 1000 bp larger than the flanking regions of the *upsEF* region (created with 44-forward/17-reverse: GGGTAGTATA TGATGGCTATAAGGTAACTG / CCGTAACAGTATATGTCTCTCCTGCAATC) confirmed the correctness of the mutants.

**Southern hybridization**

Genomic DNA (5 μg) was digested with EcoRI and PvuII and separated on a 0.8% agarose gel. Southern blotting was performed using standard procedures. DNA hybridization was performed in 50% formamid, 5 x SSC, 2% blocking reagent, 0.1% Na-lauroylsarcosin, 0.02% SDS. PCR products of *pyrEF* (using GTCATCTCTGGTCAAGTCAAGCGACGAAACTACGTGTC and GAACTTGAT-ATGAGAGAGGTTTATCCATTGC), *lacS* (using GGCATGGATCAGGTATTAGCA GAG and CCATAAG-TATGCAGTTATCAGCTCG) and *Saci_upsEF* (using GCG GGCCGCGGGATATAGAAGTTGAAAGTGGAC and CGCGCTGCAGCATTTAG GAGAAGAGCGAC). PCR products were digoxigenin-labeled with the HighPrime

Kit (Roche, the Netherlands). Detection was performed with a Luminescent Image Analyzer (FUJIFILM).

**Recombination assays for *S. acidocaldarius***

DNA exchange between *S. acidocaldarius* cells was assayed by selecting prototrophic ($Pyr^+$) recombinants of two *pyrE* mutant strains (see also Table 3). The $ups^+$ *pyrE* strains were MR31, JDS28 and JDS183 (Grogan and Gunsalus 1993; Reilly and Grogan 2002). Strain MR31 has an 18-bp deletion removing *pyrE* nt 154-171, JDS28 contains a transversion mutation (A to T) at nt 335 of the *pyrE* gene; and strain JDS183 contains a frameshift mutation (duplication of T) at nt 44. The Δ*upsEF* strain DG253 is essentially isogenic with strain JDS28; it was derived from the $pyr^+$ Δ*upsEF* SA1 strain by 5-FOA selection (Kondo *et al.* 1991), and was found by PCR and sequencing to contain a transition mutation (A to G) at *pyrE* nt 335 of *pyrE* gene. Liquid cultures were grown at 78°C and harvested at $OD_{600}$ = 0.15-0.35. Pellets were resuspended to a cell density of about 2 x $10^8$ cells/ml (Grogan 1996). UV irradiation was performed as described above. Surviving of cells was determined by serial 1:10 dilution and spread plating on uracil-supplemented xylose-tryptone plates. Phenotypic reversion of the *pyrE* mutations was assayed by plating 0.1 ml of each suspension on selective (unsupplemented xylose-tryptone) plates. Recombination was assayed by spreading a mixture representing 50 µl of each of two suspensions on selective unsupplemented xylose-tryptone plates. Plates were incubated for 5-6 days at 78°C.

**Mating assay for *S. solfataricus***

For each mating experiment a minimum volume of 60 ml of an exponentially grown *S. solfataricus* culture ($OD_{600}$ 0.2–0.4) was used. After UV treatment *S. solfataricus* strains P1 ($lacS^+/pyrEF^+$) and PH1-M16 ($lacS^-/pyrEF^-$) were mixed with equal amounts of cells. Flasks were stored in the dark for 15 min at room temperature, uracil (12.5 µg/ml final concentration) was added. Incubation was then continued without keeping the flasks in the dark at 78°C for 6 hours with mild shaking at 100 rpm. Samples were plated after dilution in Brock's basal salt pH 3, without carbon sources. Beta galactosidase activity was assayed by spraying the colonies (which appeared after 5 to 7 days) with X-Gal. To determine the number of colony forming units (cfu) without selection, cells were plated on Brock's basal salt solid media with D-arabinose (0.2%) tryptone (0.1%) and 10 µg/ml uracil as additives.

To analyze the reversion and mutation frequencies, the median of the cfu/ml with selection (5-FOA) was determined and divided by the median of the cfu/ml under non-selective conditions. To determine the recombination frequency, the median of the positive recombinants ($lacS^+/pyrEF^-$) cfu/ml with selection (5-FOA) was determined and divided by the median of the cfu/ml from all observed colonies

under non-selective conditions. To identify positive mates (*lacS*$^{+}$/*pyrEF*$^{-}$) cfu/ml with selection (5-FOA), more than 13.000 cells were counted and 788 positive colonies were identified. In the case of the control experiments in total more than 11,000 cells were counted and 5 events were identified, which correspond to the determined mutation frequency of *S. solfataricus* strain P1 of $10^{-5}$ events /cell.



## ACKNOWLEDGEMENTS

We thank Michael Wagner (Department of Microbial Ecology, University of Vienna) for generous access to his confocal laser scanning microscope. Jens Riede for help with the statistic analysis. MA was supported by an ALW grant from the Dutch Science Organization (NWO). SVA was supported by a VIDI grant from NWO and intramural funds of the Max Planck Society. DWG was supported by National Science Foundation grant MCB0543910. DT and MvW received funds from the European network SOLAR MRTN-CT-2006-033499. KS was funded by Austrian Science Fund FWF grant P20775.

# 4

## MOLECULAR ANALYSIS OF THE UV-INDUCIBLE PILI OPERON FROM *S. ACIDOCALDARIUS*

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*MicrobiologyOpen, accepted*


### ABSTRACT

Upon UV stress, hyperthermophilic *Sulfolobus* species show a highly induced transcription of a gene cluster responsible for pili biogenesis: the UV-inducible pili operon (*ups*-operon). This operon is involved in UV-induced pili assembly, cellular aggregation and subsequent DNA exchange between cells. Since the system increases the fitness of *Sulfolobus* cells after UV light exposure, we assume that transfer of DNA takes place in order to repair UV-induced DNA damages via homologous recombination. Here we studied all genes present in the *ups*-cluster via gene deletion analysis with a focus on UpsX, a protein that shows no identifiable functional domains. UspX does not seem to be structurally essential for UV-induced pili formation and cellular aggregation but appears to be important for efficient DNA transfer. In addition, we could show that pilin subunits UpsA and UpsB probably both function as major pilin subunits in the ups-pili.

## INTRODUCTION

Upon UV stress *Sulfolobus* species show a high upregulation of a gene cluster encoding proteins responsible for formation type IV pili (T4P) (Götz *et al.* 2007; Fröls *et al.* 2007): the *ups*-operon (UV-inducible pili operon of *Sulfolobus*) (Fröls *et al.* 2008). Indeed, when analyzed by transmission electron microscopy (TEM), high numbers of pili structures could be observed on the surface of UV stressed *Sulfolobus* cells from different species (Ajon *et al.* 2011). An additional phenotypic characteristic of UV stressed *Sulfolobus* cells is the formation of large cellular aggregates (Fröls *et al.* 2008), which was shown to be mediated by ups-pili in a species-specific manner (Ajon *et al.* 2011). Moreover, *Sulfolobus* cells can exchange chromosomal DNA in a pili-dependent manner which was shown to increase cellular fitness under UV stress (Ajon *et al.* 2011). Transfer of DNA therefore probably plays a role in repair of double strand breaks (DSBs) caused by UV radiation as was proposed in Ajon *et al.* (2011). Since the aggregation was also shown to be inducible by the DNA strand-break inducing agent bleomycin, the first trigger for pili formation and subsequent aggregation is thought to be the sensing DSBs in the DNA (Fröls *et al.* 2008). The mechanism behind this process is unknown. DNA exchange mechanisms among hyperthermophiles for repair of DNA have been described in more detail by van Wolferen *et al.* (2013).

Also in other organisms, T4P were shown to mediate DNA transfer (Filloux 2010). Different studies showed that the pili facilitate the uptake of extracellular DNA in competent Gram positive and Gram negative bacteria. The exact role of T4P in competence is still not well understood, but their presence is essential for successful DNA uptake (reviewed in Krüger and Stingl 2011). It has been suggested that DNA is brought close to the cell surface by binding to the pili that subsequently retract. A recent study for the first time reported the binding of a pilin subunit (minor pilin ComP) of competent *Neisseria meningitidis* to specific sequences of self-DNA suggesting that pili indeed bring DNA to the cell surface (Cehovin *et al.* 2013). Besides having a role in competence, two examples are known in which T4P are involved in a conjugative system. Firstly, the IncI1 conjugative plasmid R64 in *E. coli*, that carries the *pil* genes (Kim and Komano 1997; Yoshida *et al.* 1999; Komano *et al.* 2000) and secondly, PAPI-1 DNA which conjugates between *Pseudomonas* species and also comprises *pil* genes. The latter is located on a pathogenicity island that has been obtained via horizontal gene transfer (Carter *et al.* 2010). A role of T4P in cellular chromosomal exchange and subsequent repair of damaged DNA has never been shown before. Moreover, unlike the described bacterial T4P, the archaeal *ups* genes are not present on a conjugative plasmid or acquired via horizontal gene transfer.

The UV-induced pili system was initially studied in *S. solfataricus* (Fröls *et al.* 2008; Ajon *et al.* 2011), but because of the availability of genetic tools for manipulating the *S. acidocaldarius* genome (Wagner *et al.* 2009; Wagner *et al.* 2012), we switched to the latter organism. There is a high conservation and functional similarity among all sequenced *Sulfolobus* species. The *ups*-operon encodes five proteins: UpsX, a hypothetical protein with no conserved regions; UpsE, an ATPase; UpsF an integral membrane protein; and UpsA and UpsB, two putative pilin subunits containing class III signal peptides.

Here we have analyzed the different roles of the genes in the *ups*-cluster. The secretion ATPase UpsE and the membrane protein UpsF are, as expected, essential for the formation of ups pili. Interestingly, UpsX, is not essential for pili formation but the deletion of its gene resulted in decreased DNA exchange suggesting a role in DNA transfer. Pilin subunits UpsA and B are essential for cellular aggregation. However, as deletion mutants of either of the subunits still formed pili, it appears that a mixed structure is essential for cellular aggregation.

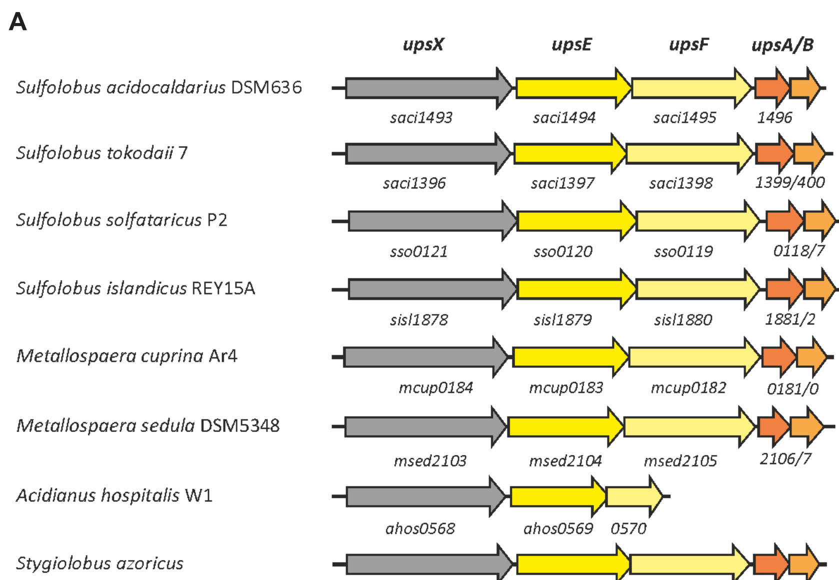


## RESULTS

### Bioinformatics and transcriptional analysis on the *ups*-operon

As described before (Fröls *et al.* 2008), the *ups*-operon encodes five proteins that are together thought to build the type IV pilus (Figure 1): UpsE, a secretion ATPase; UpsF an integral membrane protein; UpsA and UpsB, two putative pilin subunits containing class III signal peptides; and UpsX, a protein with unknown function. UpsX is a predicted cytoplasmic protein, but in contrast to UpsE, F, A and B, no specific domains could be predicted by BLAST (Altschul *et al.* 1990), SMART (Schultz *et al.* 1998) and HHpred (Söding 2005) (data not shown). An alignment of UpsX sequences revealed several conserved regions and amino acids (Figure S1). Since the bioinformatics did not predict a possible UpsX function, it was subjected to further functional analysis.

Synteny analysis (SyntTax; Oberto 2013) revealed that all sequenced Sulfolobales contain an *ups*-operon (Figure 1), including recently sequenced *Stygiolobus azoricus* (Wagner and Albers, unpublished). Though it must be noted that *Acidianus hospitalis,* member of the Sulfolobales, probably does not build functional ups-pili as no homologues of *upsA* and *upsB* could be found in or outside of the *ups*-operon. In addition, the membrane protein encoding *upsF* seems to be incomplete in this species. It is therefore likely that *A. hospitalis* lost part of its *ups*-operon throughout evolution. Deep sequencing on *S. acidocaldarius* cDNA (Wurtzel *et al.*, unpublished) revealed a transcriptional start site (TSS) in front of *upsX* (with ~10000 transcript reads), and TSSs in front of the *upsE* (160 reads) and *upsA* (133 reads) genes. These results suggest a primary TSS in front of *upsX* and



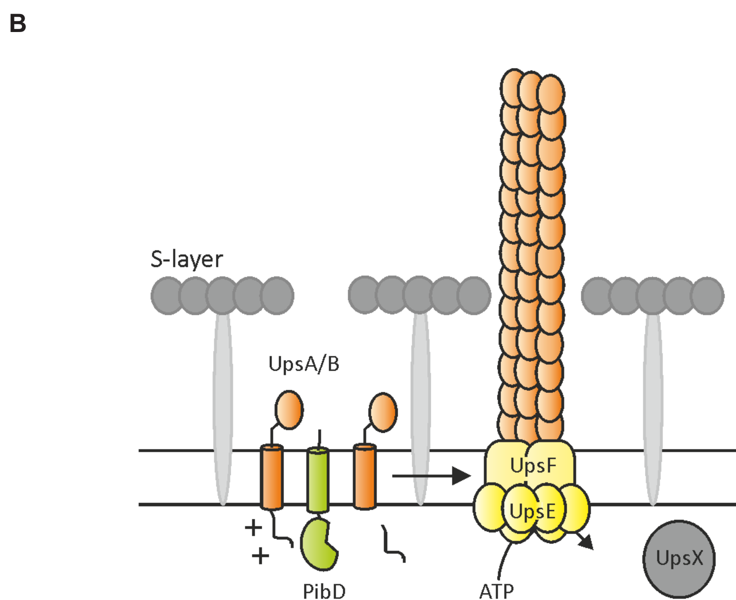




**Figure 1.** Schematic overview of the *ups*-gene cluster in different Sulfolobales. The cluster encodes: UpsX, a protein with unknown function; UpsE, a secretion ATPase; UpsF, an integral membrane protein; UpsA and B, two pilin subunits. As not all genes (especially the pilin subunits) were annotated properly, they were corrected by hand. Homology was found using SyntTax (Oberto 2013) is indicated by similar colors. The relevant portion of the genomic DNA sequence of *Stygiolobus azoricus* was annotated manually (Wagner and Albers, unpublished).

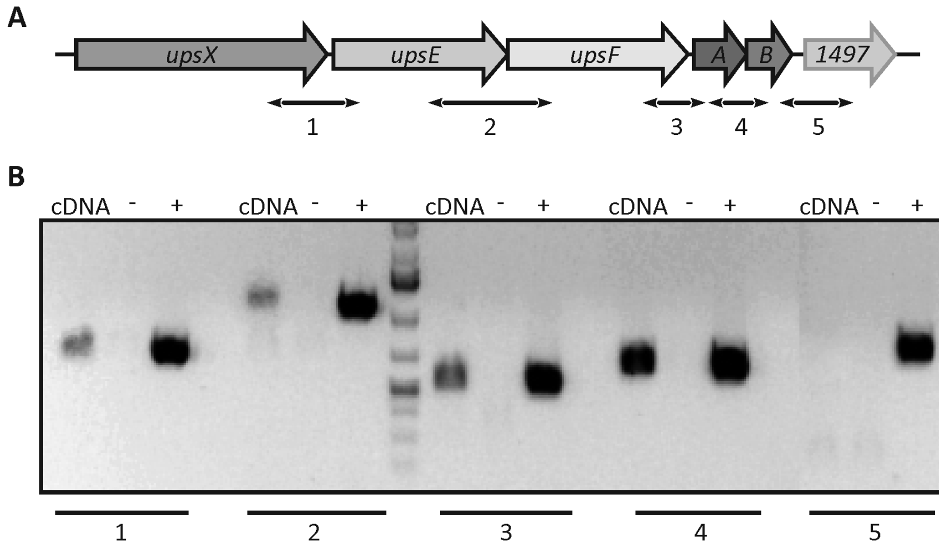



**Figure 2.** Operon mapping on the *ups*-gene cluster of *S. acidocaldarius*. (A) Gene organization of the *ups*-gene locus and downstream gene *saci1497*. Black numbered arrows indicate the regions of interest for amplification. (B) RT-PCR on cDNA from *S. acodicaldarius* MW001 induced with UV. Genomic DNA was used as positive control (+) and RNA treated with DNase as negative control (-).

secondary TSSs in front of *upsE* and *upsA*. Operon mapping using RT-PCR indeed shows that transcripts of *upsX-B* are connected and therefore probably present as one long transcript (Figure 2). This is confirmed by the observation that a deletion of both the *upsX* gene and its promoter (Δ*upsX* -40) leads to an unpiliated phenotype, whereas a clean deletion of *upsX* does not (Figure 6). Interestingly, the deep sequencing data revealed several antisense TSSs in *upsB*, which might have a regulatory function. Importantly, these deep sequencing data shows already transcription numbers without prior UV induction of the samples, indicating that the operon is expressed highly even without UV stress. The ups-system is therefore probably constitutively active. Previous microarray studies showed an upregulation of the transcription of all *ups*-genes with $\log_2$ folds of up to 3 (Götz *et al.* 2007; Fröls *et al.* 2007). By means of qPCR, this induction could be confirmed showing even higher $\log_2$ folds of between 4 and 5 (Figure 3).

## DELETION MUTANT ANALYSIS OF GENES IN THE *UPS*-OPERON

In order to obtain more insights in the individual roles of the genes in the *ups*-operon, markerless deletion mutants were created as described previously (Wagner *et al.* 2012) (Table 2). The genotypes were confirmed by PCR and sequence analysis (data not

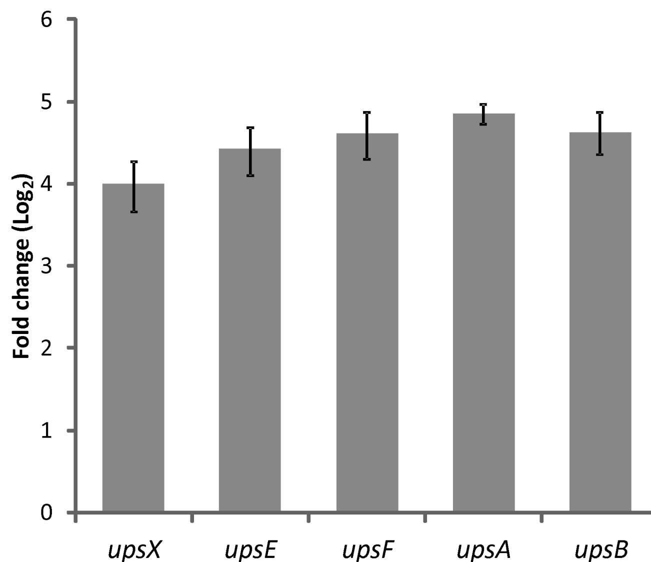



**Figure 3.** Transcriptional regulation of *ups*-genes upon UV treatment. Change of transcription levels of genes in the *ups*-operon in MW001 three hours after UV treatment (75 J/m$^2$) as measured by qRT-PCR. Differences are displayed as $Log_2$ fold changes. Used primers are summarized in Table S1.

shown). In addition, transcription levels of *ups* genes in the different deletion mutants were compared with those from wild type cells by qPCR (Figure 4). Generally, no strong polar effects could be observed on downstream genes of the deleted genes of interest. However, the deletion of *upsE* resulted in a lower transcription of *upsF*. Moreover, in an *upsF* mutant transcription of *upsA* and *upsB* was slightly reduced. The latter might possibly be due to the partial removal of the possible promoter region of *upsA* by the deletion of *upsF*. Growth curves and microscopy revealed wild-type growth and a normal cellular phenotype for all deletion mutants (data not shown). In deletion mutants of *upsE, F, A* and *B,* no UV-induced aggregation could be observed (Figure 5). This was to be expected as the secretion ATPase (UpsE), membrane protein (UpsF) and pilin subunits (UpsA/B) are all thought to be essential for pili formation. In line with this, no ups-pili formation could be observed for the *upsE* and *F* deletion strains (Ajon *et al.* 2011, Figure 6, and data not shown). Since it is unknown if and how the two predicted pilin subunits UpsA and UpsB together build up one filament, we also looked at single deletion mutants of *upsA* and *upsB*. To not confuse ups-filaments with other surface structures (Jarrell and Albers 2012; Henche *et al.* 2012), these strains were made in an archaella- and aap (archaeal adhesive) pili-less background (MW501). When the single mutants of *upsA* and *upsB* were analyzed with EM, both strains showed pili formation, although at smaller numbers. These

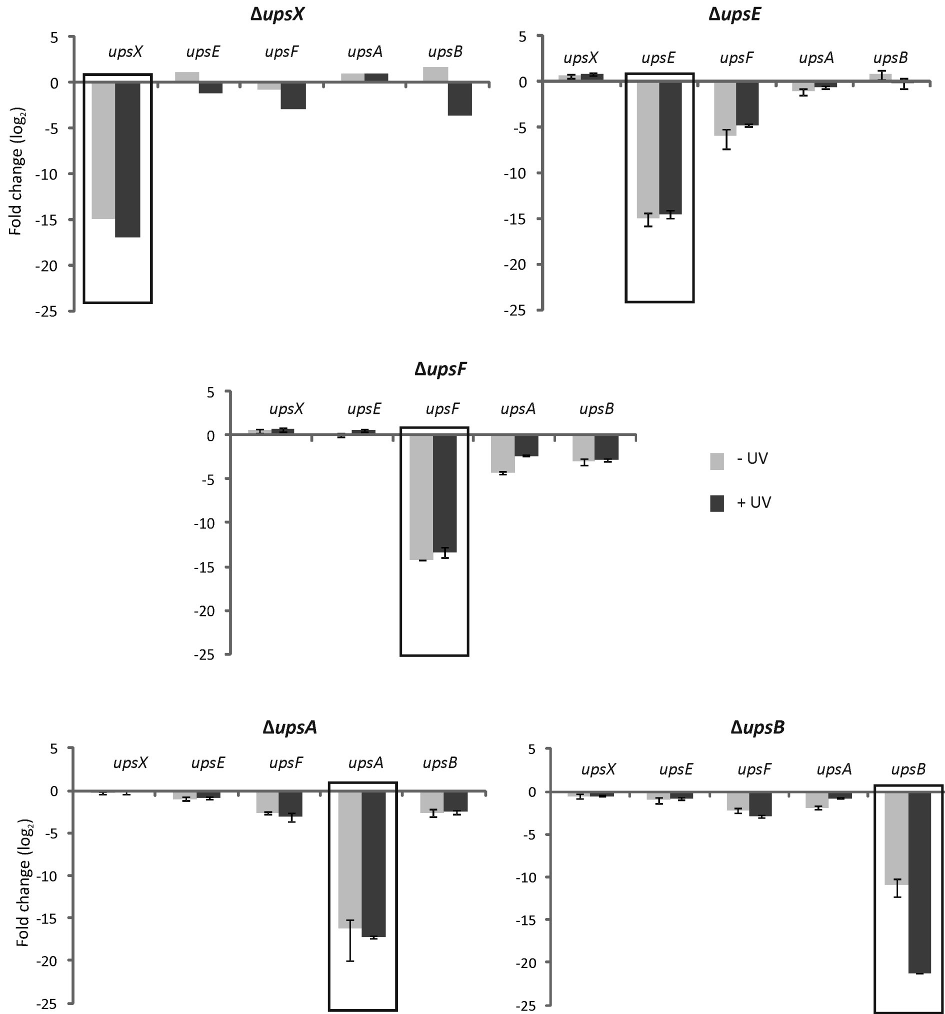



**Figure 4.** qPCR analysis comparing gene expression from *ups* mutants with that from wildtype *S. acidocaldarius*. To show the effects of *ups* deletions on neighbouring gene expression, RNA from deletion mutants, treated with or without UV (light and dark grey, respectively), was isolated and converted to cDNA. cDNA was subjected to qRT-PCR analysis using primers specific for indicated *ups*-genes. Strains used were: Δ*upsX* (MW115), Δ*upsE* (MW109), Δ*upsF* (MW110), Δ*upsA* (MW106) and Δ*upsB* (MW107). Shown are $Log_2$ fold change values in expression compared to MW001. Bars marked with a box are the deleted gene of the corresponding mutant. The means and standard deviations of biological replicates are shown.

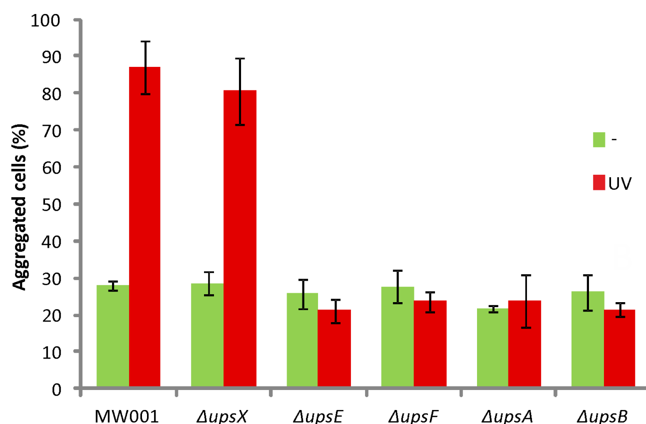



**Figure 5.** Aggregation assays with *ups* deletion strains. Light microscopy analysis of aggregation of the different *ups* deletion strains 3 hours after induction with UV (75 J/m²). Strains used were: Δ*upsX* (MW115), Δ*upsE* (MW109), Δ*upsF* (MW110), Δ*upsA* (MW106) and Δ*upsB* (MW107).

**Figure 6.** Transmission electron microscopy on UV induced *S. acidocaldarius* mutants. Pili formation of wild type *S. acidocaldarius* cells (MR31), Δ*upsA* (MW138), Δ*upsB* (MW140) Δ*upsX* -40 (including promoter region, GA06) and Δ*upsX* (MW115). *Scale bar* 100 nm

results suggest that both subunits are capable to form filaments but that for efficient pili formation both genes are needed likely resulting in mixed subunit pili.

Interestingly, the Δ*upsX* strain shows wild-type cellular aggregation upon UV treatment and also the ups-pili are wild type-like (Figure 5 and 6). UpsX does therefore not seem to have an essential role in the pili formation or cellular recognition (aggregation).



**Chromosomal marker exchange**

To study a putative role of UpsX in DNA exchange between *Sulfolobus* cells, DNA transfer assays were performed. Series of auxotrophic *ups/pyrE* double mutants were used in mating experiments (Table 2). Two parental strains were mixed together and upon exchange of chromosomal DNA, *pyrE* mutations could be restored via homologous recombination resulting in prototrophic colonies as described previously (Ajon *et al.* 2011). For each experiment the results were normalized, taking the mixture of wt1 (UV)*wt2 (C) as 100% (Figure 7). A mixture of two ups wild type strains resulted in the formation of recombinants (Figure 7, wt1*wt2, green bar), indicating DNA exchange between the two strains and confirming a constitutive activity of the system, even without UV stress. Moreover, upon induction of one of the two strains with UV light, exchange of DNA increased four

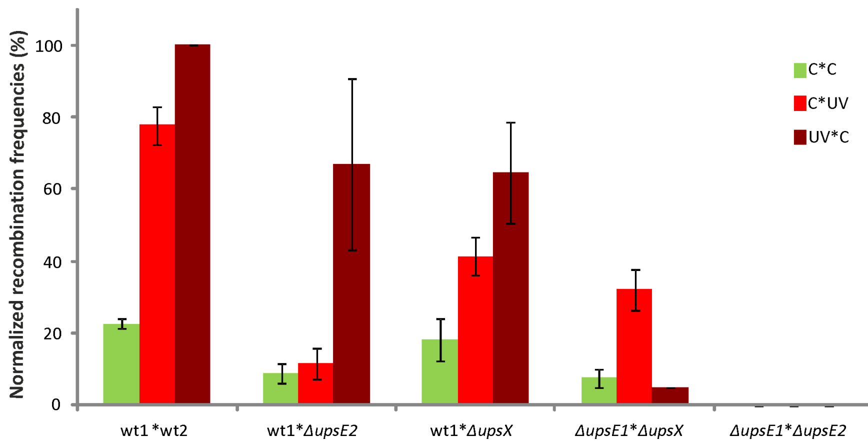



**Figure 7.** Recombination frequencies of mating experiments with *S. acidocaldarius* wild-type and mutants strains. Two different strains treated with (UV) or without (C) UV, were mixed in different combinations and plated on selective media. All strains contained mutations in the *pyr*E gene (involved in *de novo* uracil biosynthesis) located at different positions, such that recombination between two strains can restore the wild-type phenotype. Strains used were: wt1 (MR31), wt2 (JDS183,) Δ*upsE1* (SA1), Δ*upsE2* (DG253), Δ*upsX* (GA09). Bars represent the average of 3 independent mating experiments each, every experiment was normalized to wt1 (UV) * wt2 (C) as 100%.

to five-fold (Figure 7, wt1*wt2, red bars). An *upsE* deletion strain did not support transfer of DNA when treated with UV light (Figure 7, wt1*Δ*upsE2*, first red bar). Only when the wild-type strain (wt1) was induced with UV in this mixture, a significant increase of DNA exchange could be observed, showing that only one of the two strains needs to assemble pili for DNA exchange to occur. A mixture of two Δ*upsE* strains resulted in no DNA transfer (Δ*upsE1**Δ*upsE2*). These results confirmed the previously observed essential function of ups-pili for DNA exchange between *Sulfolobus* cells (Ajon *et al.* 2011). However, when mixing the wt2 with UV-induced Δ*upsX*, still a higher amount of recombinants were formed than without UV induction, although this increase was only about 50% of that of a wt1*wt2 mixture. Similarly, a mixture of Δ*upsX* with Δ*upsE* resulted in a significantly lower increase of DNA exchange upon UV induction of the Δ*upsX* strain. These results imply that UpsX plays a role in the process of UV-induced DNA transfer between *Sulfolobus* cells that it is not essential. Possibly, UpsX plays a direct or indirect role in DNA transfer or processing of incoming/outgoing DNA.





### ***In vivo* protein levels and localization of UpsX**

To localize UpsX in the cells, *S. acidocaldarius upsX* was genomically tagged with a C-terminal HA-tag (MW101, Table 2). This strain was induced with UV and samples were taken 0, 1, 2 and 3 hours after UV irradiation. Cytosol (C) and membranes (M) were subsequently separated using ultracentrifugation. Western blotting analysis on the different fractions confirmed that UpsX-HA migrates with the expected size of around 79 kDa on SDS-PAGE, with some smaller degradation products present in the cytosol fractions (Figure 8). The 55 kDa protein was confirmed to be unspecific as it was also present in the negative control (-, lysate of MW001).

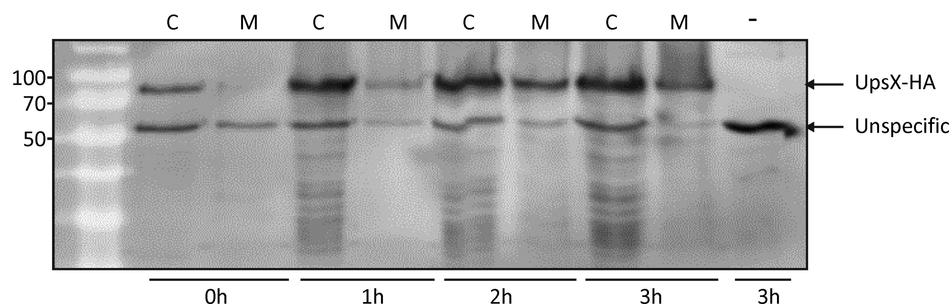



**Figure 8.** Time course of UpsX-HA expression upon UV treatment in MW101 cells. Membrane (M) and cytosol (C) fractions from MW101 (C-terminally HA tagged UpsX) 0h, 1h, 2h and 3h after UV induction were separated on SDS-PAGE. As a negative control, lysate from MW001 was used (-) showing a clear unspecific band, but no band at the size of UpsX-HA (79 kDa). Proteins were visualized by Western blotting using α-HA primary antibodies.

Therefore it was used as an internal loading standard. After UV induction, a clear increase of UpsX protein in line with the transcriptional response could be observed (Figure 3). Interestingly, without UV induction, UpsX was found only in the cytosol while after UV stress, a significant fraction of about 35% of the protein seemed to localize to the membrane (Figure 8).

## DISCUSSION



T4P have shown to be involved in numerous functions both in bacteria as well as in archaea. In Sulfolobales, different type IV pili have been described, their functions include: motility, attachment to surfaces, biofilm formation, and DNA exchange. In *S. acidocaldarius*, three different T4P involved in these functions can be found: aap pili (Henche *et al.* 2012), archaella (Jarrell and Albers 2012) and ups-pili (Fröls *et al.* 2008; Ajon *et al.* 2011). Previous studies have shown the involvement of ups-pili in UV-induced cellular aggregation and DNA exchange. This mechanism is unique to the Sulfolobales and is proposed to be involved in repair of DNA double strand breaks (Fröls *et al.* 2008; Ajon *et al.* 2011). Here we have studied the individual genes of the *S. acidocaldarius ups*-gene cluster in more detail using bioinformatics, transcriptional analyses, deletion mutant analyses and localization experiments.

Synteny analysis revealed that all Sulfolobales but no other species contain an *ups*-cluster encoding: UpsX, a protein with unknown function; UpsE, a secretion ATPase; UpsF, a membrane protein; and UpsA and B, two class III signal peptide containing pilin subunits. Expression and induction of this operon has been shown for a number of *Sulfolobus* species (Ajon *et al.* 2011). The ups-system of *Acidianus hospitalis* is almost certainly not active as it lacks both pilin subunits and part of the *upsF* gene, suggesting that it lost these genes during evolution. A possible explanation for this loss might be that *Acidianus* species do not encounter as much UV light as other Sulfolobales and therefore have slower mutation rates. For instance *A. hospitalis* is a facultative anaerobe (unlike obligate aerobic *Sulfolobus* and *Metallosphaera* species) may thus grow in deeper, darker, areas of hotsprings (Giaveno *et al.* 2013) being less exposed to UV. In other Sulfolobales, the *ups*-genes can be readily deleted with no apparent effect on vegetative growth. However, the presence of ups-pili does have a clear beneficial effect on the vitality of *Sulfolbus* cells upon severe DNA damaging conditions (Ajon *et al.* 2011). The ups-system might therefore have evolved to conquer extremely DNA damaging conditions such as high temperatures, low pH values and high UV doses. The increased exchange of DNA would make regular homologous recombination more efficient by increasing the chances of having a homologous template.

Operon mapping confirmed that the *ups* cluster of *S. acidocaldarius* is transcribed as one transcript demonstrating that it is truly an operon. Indeed, a deletion of

the primary promoter region in front of *upsX* aborted pili formation whereas a clean deletion of *upsX* has no effect. Deep sequencing data moreover showed the presence of additional TSSs within the operon in front of *upsE* and *upsA* (Wurtzel *et al.* unpublished). Secondary promoter elements were also found in the *S. acidocaldarius* archaella operon (Lassak *et al.* 2012) and might be essential to fine-tune the stoichiometry of the different proteins within the ups pili: e.g. much more pilin subunits will be needed than membrane proteins involved in the assembly of a functional pilus. The strong upregulation of the *ups*-operon upon UV light was confirmed with qRT-PCR.

Deletion mutants of genes in the *ups*-operon revealed that *upsE* and *upsF* are individually essential for pili formation as well as aggregation further expanding on the observation with the *upsEF* double deletion mutant (Ajon *et al.* 2011). Deletion mutants of the pilin subunit genes *upsA* and *upsB* no longer showed UV-induced aggregation. Interestingly though, either of the strains still formed ups-pili, but in lesser numbers. These results suggest that UpsA and UpsB are both major pilin subunits that in wild-type cells might form a mixed pilus structure, this in contrast to bacterial T4P systems which were so far found to have only one major pilin subunit (Ayers *et al.* 2010; Giltner *et al.* 2012). In bacteria minor and major pilins have been implicated in adherence to various different surfaces, cells from the same species and host tissues (reviewed by Giltner *et al.* 2012) Also ups subunits are thought to be involved in strain specific recognition and interaction between cells from the same species (Ajon *et al.* 2011). Preliminary data suggests that a specific region in the pilin subunit UpsA is responsible for the recognition glycosylated S-layer proteins from cells from the same species (van Wolferen and Albers, unpublished).

UpsX is a protein with so far unknown function, but it is highly conserved in all species that contain an *ups*-operon suggesting that it fulfils an essential role UV-dependent DNA transfer. Intriguingly, however, a deletion mutant of *upsX* still formed ups-pili and while the UV-induced cellular aggregation was comparable to wild-type. UpsX does therefore not seem to play a structural role in the ups-pilus nor is it essential for cellular recognition or assembly of the pili. Possibly other functions of UpsX would be DNA transfer, and indeed, a UV-induced *upsX* deletion mutant contributed less to DNA transfer compared to the wild type strain. These data indicate that UpsX promotes DNA exchange but it is not essential for this process. Interestingly, a significantly larger portion of the UpsX protein localized to the membrane fraction in time upon UV exposure. Possibly, UpsX promotes DNA exchange at the membrane via a yet unknown mechanism. Future biochemical experiments might provide more insights into the actual function of UpsX.

## EXPERIMENTAL PROCEDURES

### Culture conditions

*Sulfolobus acidocaldarius* strains MW001, MR31, JDS183 and derived mutants were grown aerobically at 78°C in basic Brock medium (Brock *et al.* 1972) supplemented with 0.1% NZ amine, 0.2% dextrin and 20 µg/ml uracil and adjusted to pH 3.5 with sulfuric acid. For solid media the medium was supplemented with 1.5% gelrite. Plates were incubated for 5-6 days at 78°C. *E. coli* competent cells DH5α, ER1821 (NEB), used for respectively cloning and methylation of plasmid DNA, were grown in LB medium (10 g/l tryptone; 5 g/l yeast extract; 10 g/l NaCl) at 37°C supplemented with the appropriate antibiotics. Growth of cells was monitored by optical density measurements at 600 nm.

### UV treatment, aggregation assays

UV light treatment was performed as described in Fröls *et al.* (2008). 10 ml culture ($OD_{600}$ 0.2-0.3) was treated with a UV dose of 75 J/$m^2$ (254 nm, Spectroline, UV crosslinker) in a plastic petri dish. Afterwards cultures were put back at 78°C for 3 hours. Samples taken at different time points were analyzed with phase contrast microscopy, survival rate assays and electron microscopy. To quantify aggregated cells after induction with UV, 5 µl of cell culture (diluted to OD 0.2) was spotted on a microscope slide covered with a thin layer of 1% agarose in Brock minimal medium. A coverslip was added when the drop had dried. Cells were visualized with phase contrast microscopy. Free and aggregated cells (≥ 3) were counted for at least three fields per strain using ImageJ cell counter. Percentages of cells in aggregates were subsequently calculated.

### Deleting/tagging genes in *S. acidocaldarius*

To construct deletion and gene replacement strains, up- and downstream flanking regions of the genes of interest or place to insert a tag (approximately 600 bp) were amplified with primers listed in Table S1. Primers were designed according to the genomic sequence of *Sulfolobus acidocaldarius* DSM 639. Overlap PCR was performed to connect the up- and downstream fragments. The PCR product was subsequently cloned into pSVA406, resulting in the plasmids summarized in Table 1. The plasmids were methylated in *E. coli* ER1821 containing pM.EsaBC4I (NEB) (Kurosawa and Grogan 2005) and transformed into *S. acidocaldarius* MW001/MR31 (Wagner *et al.* 2012). Integrants were selected on plates lacking uracil and grown in 24-well plates for 2 days in the same medium. Subsequently cultures were plated and grown for 5 days on second selection plates containing uracil and 100 µg/ml 5-FOA to select for clones in which the plasmid looped out by homologous recombination. Obtained colonies were tested by PCR for successful deletion/replacement of the genes. Correctness of strains was

**Table 1.** Plasmids used during this study.

| Plasmid | Description | Source/reference |
|---|---|---|
| pMA08 | Deletion plasmid for *ΔupsX*+promoter region *(Δsaci1493, -40*bp*)* | This study |
| pSVA406 | Backbone of deletion plasmids | (Wagner *et al.* 2012) |
| pSVA180 | Deletion plasmid for *ΔaapF (Δsaci2318)* | Gosh and Albers, unpublished |
| pSVA329 | Deletion plasmid for*ΔflaI* (*saci1173* Δbp 1-672) | Gosh and Albers, unpublished |
| pSVA1819 | Deletion plasmid for *ΔupsX (Δsaci1493)* | This study |
| pSVA1801 | Deletion plasmid for *ΔupsA (Δsaci1496)* | This study |
| pSVA1802 | Deletion plasmid for *ΔupsB (Δsaci1496b)* | This study |
| pSVA1805 | Deletion plasmid for *ΔupsF (Δsaci1495)* | This study |
| pSVA1832 | Plasmid to add C-term HA-tag on *upsX (saci1493)* | This study |

confirmed by DNA sequencing. Strains that were made during this study are listed Table 2.

**qPCR and operon mapping on *S. acidocaldarius* cDNA**

To compare expression of the *ups*-operon and its downstream genes from different strains (UV/not-UV-induced), RNA was isolated from 10 ml cultures (from MW001 and mutants, appendix 3), using TriFast™ (Peqlab). DNA was subsequently degraded by incubating the RNA with DNAseI (RNAse free, Fermentas) according to the manufacturer's protocol. Proper DNA degradation was confirmed by performing a PCR with primer pair 2033 + 2087 (Table S1) on the RNA. cDNA synthesis was performed on 1 µg of RNA with the First strand cDNA Synthesis Kit (Fermentas). Random primers were used and the manufacturer's protocol was followed. qPCR was performed using the Maxima SYBR Green/ROX qPCR mastermix. qPCR primers were designed for *saci1493 – saci1496b (upsX-upsB)*, they have a melting temperature around 60°C and give a product of 80 – 150 bp in length (primers 2073 – 2082, Table S1). As a control, a primer for an *lrs14* gene that was not found to be differentially expressed after induction with UV in microarray studies was used. Control qPCR was performed according to the manufacturer's instructions. The obtained CT values were used to compare non-UV induced with UV-induced expression of the tested genes. Moreover expression was compared between MW001 and deletion strains. Differences in expression were displayed as log2 folds.

To see if the genes in the *ups* gene cluster are present as an operon, isolated cDNA of UV-induced *S. acidocaldarius* MW001 was used to amplify intergenic

**Table 2.** Strains used during this study.

| Strain | Background strain | Genotype | Source/ reference |
|---|---|---|---|
| MR31 (wt1) | *S. acidocaldarius* DSM639 | Δ*pyrE* (Δbp 154-171 ) | Reilly and Grogan 2001 |
| JDS183 (wt2) | *S. acidocaldarius* DSM639 | Δ*pyrE* (2xbp 44 ) | Grogan and Hansen 2003 |
| SA1 (Δ*upsE1*) | *S. acidocaldarius* MR31 | Δ*upsE*+Δ*upsF* | Ajon *et al.* 2011 |
| DG253 (Δ*upsE2*) | *S. acidocaldarius* SA1 | Δ*upsE*+Δ*upsF*, Δ*pyrE* (Δbp 335) | Ajon *et al.* 2011 |
| GA06 | *S. acidocaldarius* MR31 | Δ*upsX*-40 bp | This study |
| GA07 | *S. acidocaldarius* MR31 | Δ*upsX* | This study |
| GA09 (Δ*upsX)* | *S. acidocaldarius* GA07 | Δ*upsX* Δ*pyrE* (Δbp 28) | This study |
| MW001 | *S. acidocaldarius* DSM639 | Δ*pyrE* (Δbp 91–412) | Wagner *et al.* 2012 |
| MW101 | *S. acidocaldarius* MW001 | *upsX*+ C-term HA | This study |
| MW106 | *S. acidocaldarius* MW001 | Δ*upsA* | This study |
| MW107 | *S. acidocaldarius* MW001 | Δ*upsB* | This study |
| MW109 | *S. acidocaldarius* MW001 | Δ*upsE* | Wagner *et al.* 2012 |
| MW110 | *S. acidocaldarius* MW001 | Δ*upsF* | This study |
| MW115 | *S. acidocaldarius* MW001 | Δ*upsX* | This study |
| MW138 | *S. acidocaldarius* MW501 | Δ*upsA*, Δ*flaI* (Δbp 1-672), Δ*aapF* | This study |
| MW140 | *S. acidocaldarius* MW501 | Δ*upsB*, Δ*flaI* (Δbp 1-672), Δ*aapF* | This study |
| MW501 | *S. acidocaldarius* MW001 | Δ*flaI* (Δbp 1-672), Δ*aapF* | Gosh and Albers, unpublished |

regions between *upsX* and *E*; *E* and *F*; *F* and *A*; and *A* and *B* using primer pairs: 2073 + 2076; 2020 + 2013; 2077 + 2080; and 2038 + 2082, respectively (Table S1).

**DNA transfer assays**

DNA transfer between *S. acidocaldarius* cells was assayed by selecting prototrophic (Pyr$^+$) recombinants of two *pyrE* mutant strains. The *ups*$^+$ *pyrE* strains were MR31 (wt1) and JDS183 (wt2). Strain MR31 has an 18-bp deletion in *pyrE* (nt 154-171). Strain JDS183 contains a frame shift mutation (duplication of T) at nt 44. The Δ*upsE* strains were SA1 (Δ*upsE1*) and DG253 (Δ*upsE2*). SA1 was derived from background strain MR31, DG253 contains a transition mutation (A to G) at *pyrE* nt 335. The Δ*upsX* strain was GA09, which contains a 1 bp (A) deletion in 28 nt in *pyrE* gene. Liquid cultures were grown at 78°C and harvested at $OD_{600}$ 0.15 to 0.35. Pellets were resuspended to a cell density of about 2 x 108 cells/ml. UV irradiation was performed as described before (Ajon *et al.* 2011). Recombination was assayed

by spreading a mixture with 50 µl of each of two suspensions on selective plates without uracil. Plates were incubated for 5-6 days at 78°C as was described previously (Ajon *et al.* 2011). For each experiment the results were normalized, taking mixture wt1(UV)*wt2(C) as 100%.

**Electron microscopy analysis**

UV-induced pili in *S. acidocaldarius* cells and derived mutants were observed with transmission electron microscopy. Specimens were negatively stained with 2% uranyl acetate on carbon-coated copper grids. Microscopy was performed with a Philips CM10 electron microscopy operated at 120 kV. Images were recorded using a Gatan 4K CCD camera at different magnifications.

**Western blot analysis of HA-tagged UpsX**

UV irradiation on MW001 and MW101 was performed as described before above. Subsequently 20 ml of control and UV-treated samples were harvested at 0, 1h, 2h and 3h time points and resuspended in 50mM HEPES buffer pH 8, containing 150mM KCl. Cells were broken by 10 cycles of sonication. Unbroken cells were removed by low spin centrifugation (8000 rpm x 10 min, 4°C). Cytoplasmic and membrane fractions were obtained during ultracentrifugation step (70000 rpm x 30 min, 4°C). Isolated membranes were solubilised in 50mM HEPES/150mM KCl buffer pH 8, supplemented with 1% Triton X-100 for 45 min at room temperature. All samples were run on a 12% SDS-PAGE gel. Western blot was performed on polyvinylidene difluoride (PVDF) membrane, blocked overnight with 2% I-Block in PBST buffer and followed by incubation with 1:100 primary anti-HA antibodies (Sigma) and 1:30000 secondary anti-Rabbit IgG-Alkaline Phosphatase antibodies (Sigma). Chemiluminescence signal was obtained by CDP-Star (Roche) on Roche Lumi-imager. Intensities of the bands were quantified using ImageJ.

## ACKNOWLEDGEMENTS

We thank O. Wurzel for access to unpublished deep sequencing data. M.vW was supported by a grant from the German Science Foundation (DFG, AL1206/3-1). M.A was supported by an ALW grant from the Dutch Science Organization (NWO). S.VA received support from intramural funds of the Max Planck Society.

# 5

## SUMMARY AND CONCLUSIONS

## INTRODUCTION

Archaea as a third domain of life (Woese *et al.* 1977) represent an interesting object to study aspects of evolution, adaptation and stress response mechanisms. In size and shape, Archaea are similar to Bacteria, however many genes and mechanisms involved in information processing, including DNA replication, transcription and translation, are more related to Eukarya than Bacteria. Archaea also harbor many unique (metabolic) properties that are not found in the other two domains of life, like unusual pathways and enzymes of central carbohydrate metabolism (Siebers and Schönheit 2005). Many Archaea live in a harsh and extreme environment, like hot springs (hyperthermophiles) or salt lakes (halophiles), although their distribution is not only limited to extreme environments which became evident with the discovery of mesophilic Archaea. In the meantime, the methods have been developed to grow Archaea under laboratory conditions, while for some even genetic tools for gene deletion and protein expression are available. These advances make Archaea especially interesting group of microorganisms to study.

One of the best characterized archaeal members are the *Sulfolobus* sp. These belong to the thermoacidophilic kingdom Crenearchaeota and thrive mainly in volcanic springs at optimal temperatures around 80°C and low acidic pH (pH 2–3). The most famous strains from Sulfolobaceae family include *Sulfolobus acidocaldarius*, the first hyperthermophilic organism discovered in Yellowstone National Park (Brock *et al.* 1972) and *Sulfolobus solfataricus* P2 from the solfatara fields at the volcano Vesuvius close to Naples, Italy (Zillig *et al.* 1980). The genome of *Sulfolobus solfataricus* was the first to be sequenced within the Sulfolobales (She *et al.* 2001), and several other genomes followed soon. Since *Sulfolobus* strains are rather easy to cultivate in a laboratory, they are now the model organism for Crenearchaeota from which *S. solfataricus* and *S. acidocaldarius* were used for the work described in this thesis.

Throughout evolution, adaptation to extreme environments has been crucial for hyperthermophilic organisms. Transfer of DNA, especially horizontal gene transfer (HGT) has been shown to be an important factor in microbial genome evolution. Moreover, exchange of DNA plays an important role in DNA repair via homologous recombination, especially under the DNA damaging highly thermophilic conditions. For Archaea and Bacteria three basic mechanisms for DNA transport have been characterized: natural transformation, conjugation and transduction. The main mechanism involved in horizontal gene transfer is conjugation. It is the unidirectional transfer of genetic material between cells where cell-to-cell contact or a bridge-like connection is required. The different DNA exchange mechanisms in hyperthermophilic Archaea and Bacteria are reviewed in **Chapter 1** of this thesis. Furthermore, the review emphasizes the role

of DNA exchange in survival and adaptation of hyperthermophilic organisms to extreme conditions. The following chapters describe experimental studies on mechanisms of DNA transfer found in Sulfolobales that involves type IV pili and is UV-induced. The complex transcriptional response to UV irradiation found both in *S. solfataricus* and S. *acidocaldarius* (Fröls *et al.* 2007; Götz *et al.* 2007) led to the identification of UV-induced pili. Formation of these pili at the cell surface and subsequent cellular aggregation was shown to enhance DNA exchange among *Sulfolobus* cells. This thesis describes the first reports on this UV-inducible DNA exchange system in the hyperthermophilic *Sulfolobus* species.



## UV-INDUCIBLE AGGREGATION AND TYPE-IV PILI OF SULFOLOBALES



The pili biogenesis operon harbors among the strongest UV-induced genes in Sulfolobales. This system is termed the *ups*-operon (UV-inducible pili operon of *Sulfolobus*) and consists of five genes that encode the structural components of a pilus assembly system including the pilin subunits. These are an ATPase (UpsE), two pre-pilin subunits (UpsA and B), a transmembrane protein (UpsF) and a protein of unknown function (UpsX). This operon was found to be essential for the UV-induced production of type IV pili. Pili could be visualized by transmission electron microscopy on the cell surface of UV-induced cells. In addition, the cells aggregated into clusters of numerous cells upon UV-treatment. Different conditions were investigated to characterize these UV-induced filaments and the aggregation phenomenon in *S. solfataricus* (**Chapter 2**). The recently developed genetic system (Albers and Driessen 2008) for gene inactivation was used to target the putative secretion ATPase UpsE (SSO0120). This Δ*upsE* strain was no longer able to produce pili on its cell surface, but was also found to be defective in the UV-induced cellular aggregation. Pili of *S. solfataricus* were found to be straight filaments that are variable in length but with a uniform diameter of around 10 nm. They are clearly different and more numerous than the *S. solfataricus* archaella (previous termed flagella). The pilus filaments appeared on the cell surface as a response to UV stress around 1 h after UV irradiation. Up to 80% of cells in the culture formed cellular aggregates that are highly dynamic. Maximal aggregation was reached at 6-8h after UV treatment, followed by a slow disaggregation. The number of aggregates was found to be dependent on the UV dose and most cells were killed at high UV doses (200 or 1000 J/m$^2$). Interestingly, cellular aggregation could be also induced by DNA double strand breaks-inducing agents, like bleomycin and mitomycin C. However, other stress factors such as changes in pH or temperatures did not result in cellular aggregation. These results suggested that aggregation and pili formation are interlinked processes possibly allow the recombinational

repair among chromosomes of aggregating cells. However, a detailed mechanism of the UV-induced aggregation phenomenon and the structural features of the cells contained in these aggregates are still unknown.

Further investigations on the UV-inducible type IV pilus system were continued with the genetically more accessible *S. acidocaldarius* (**Chapter 3**). Nowadays, *S. acidocaldarius* is genetically easier accessible as deletion mutants are readily created by double crossover recombination events (Wagner *et al.* 2012; Wagner *et al.* 2009). To explore the specificity of the cell-to-cell recognition between UV-induced *Sulfolobus* cells, aggregation was analyzed by fluorescence *in situ* hybridization. Clearly, a species-specific recognition process was observed. The UV-induced pili were investigated in different *Sulfolobus* strains and the filaments were found to differ in length between *S. solfataricus*, *S. acidocaldarius* and *S. tokodaii*., *S. acidocaldarius* produced the shortest and the most abundant ups-pili. Probably the differences in pilus morphology between *Sulfolobus* strains resulted from different amino acid sequences of the individual pilin subunits.



## UV-INDUCIBLE DNA EXCHANGE IN SULFOLOBALES

Cellular aggregation in the thermoacidophilic crenarchaeote *Sulfolobus* sp. was at an earlier stage already linked to conjugative DNA transfer (Schleper *et al.* 1995). Moreover, UV stimulation was shown to greatly increase the exchange of chromosomal markers in *S. acidocaldarius* (Schmidt *et al.* 1999). However, the mechanism and the genes responsible for this DNA transfer remained unknown. Thermoacidophilic *Sulfolobus* sp. thrive in an environment of constant stress and DNA damaging conditions with potentially high UV doses. Under such conditions, special DNA repair mechanisms may be necessary to maintain cell viability. The microarray data from the UV-specific transcriptional response in *Sulfolobus* sp. did not identify genes involved in direct DNA repair (Fröls *et al.* 2007; Götz *et al.* 2007). **Chapter 3** of this thesis describes a unique DNA exchange phenomenon observed during UV-induced cellular aggregation, a process that is dependent on type IV pili. Studies presented in **Chapter 2** had raised the important question about the function of UV-induced pili-mediated aggregation. To investigate the role of this process mating experiments were performed (Hansen *et al.* 2005). Cellular aggregation was shown to induce the exchange of chromosomal markers with recombination frequencies up to three orders of magnitude higher than those of non-UV-treated cells. Also strains with a deletion of pili genes that could not produce pili and that were also defective in cellular aggregation were examined in the mating experiments. Importantly, these strains showed a reduced survival rate upon UV exposure as compared to the parental strain. The pili deletion strains (Δ*upsEF*) still yielded recombinants when mated with a wild-type partner

provided that the latter one was UV treated. However, when the two mating partners both lacked the pili, exchange of DNA markers was no longer observed. Taken together, these data demonstrated a novel role for type IV pili which mediate cellular aggregations thereby allowing DNA exchange with the purpose to repair the UV damaged DNA (Figure 1).

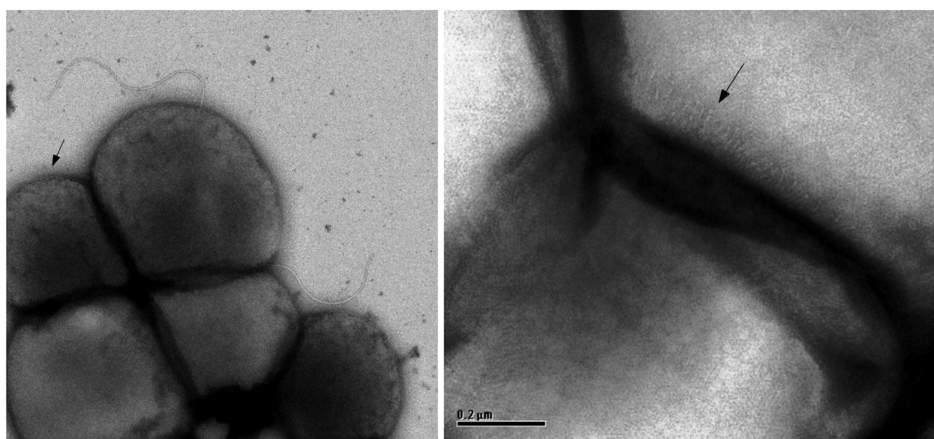



**Figure 1.** Electron micrograph of wild type *Sulfolobus acidocaldarius* cells present in cluster upon UV treatment. (A) Ups-pili are formed on the surface of UV induced cellular aggregates 3 hours after UV irradiation. (B) UV-induced pili were observed also between the cells in the aggregates. Pili centers are indicated by the arrows.

## MOLECULAR ANALYSIS OF *UPS*-OPERON OF *S. ACIDOCALDARIUS*

The secretion ATPase (UpsE) and transmembrane protein (UpsF) were already shown to be necessary for ups-pili biogenesis, cellular aggregation and for DNA exchange in the UV irradiated *Sulfolobus* cells (**Chapter 2** and **Chapter 3**). The remaining genes from the *ups*-operon of *Sulfolobus* (*upsX*, *upsA* and *upsB*) were analyzed in **Chapter 4** of this thesis using bioinformatics, transcriptional analysis, gene deletion and localization experiments. A special effort was directed to characterize and define the role of UpsX, a protein with unknown function that lacks conserved domains. UpsX is however a highly conserved protein in all *Sulfolobus* sp. The transcription levels of *ups*-genes in all deletion mutants were analyzed. All gene inactivation strains (Δ*upsE*, *F*, *A* and *B*) were defective in cellular aggregation upon UV treatment, except for the Δ*upsX* strain. Also and as expected, the ups-pili were not assembled in the Δ*upsE* and Δ*upsF* cells, as the ATPase and membrane protein are essential for type IV pili biogenesis. Interestingly, the single

mutants of the pilin subunits, *upsA* and *upsB*, both still showed pili formation, however, compared to the wild-type cells, these pili were assembled in very low amounts. Likely, both pilin subunits are part of the ups-pili. Remarkably, the *upsX* deletion strain showed a different phenotype. In contrast to other genes from the *ups*-operon, UpsX does not fulfill an essential role in the pili formation or cellular aggregation, and still shows the wild-type features in these processes. However, a mutant lacking both the *upsX* genes and its promoter region no longer formed pili filaments. Deep sequencing analysis of *S. acidocaldarius* cDNA showed not only a transcriptional start site (TSS) in front of the *upsX* gene, but also additional TSSs in front of *upsE* and *upsA*. This suggests that secondary transcription start can be used to fine-tune the stochiometry of the pilin proteins within the ups-pili. To analyze the role of UpsX in DNA exchange between *Sulfolobus* cells, the assay from **Chapter 3** was used. In a conjugation assay wherein the wild type strain was mixed with UV-induced Δ*upsX*, the number of recombinants was higher than without UV irradiation but only at about a 50% level of that observed with a mating experiment using wild type strains only. This suggested that UpsX plays a role in maintaining efficient DNA transfer between *Sulfolobus* cells; albeit non-essential. Upon UV treatment, a large portion of the cellular UpsX localizes to the membranes, while UpsX is predicted to be a cytoplasmic protein. Possibly, UpsX is involved in the DNA transfer mechanism although currently it is not known how it acts in UV-induced conjugation.

## OUTLOOK AND CONCLUDING REMARKS

Type IV pili are filamentous structures that are involved in several cellular functions in many bacteria and archaea. They have mostly been implicated in cell motility, surface adhesion, biofilm formation and DNA uptake. In this thesis, type IV pili were found to mediate UV-induced chromosomal marker exchange and cellular aggregation in hyperthermophilic *Sulfolobus* species. Pili could be induced under laboratory conditions at high energy short wave UV-C light (100-280 nm) with a wavelength 254 nm. In nature, sunlight is composed of around 96% of long wave UV-A (315-400 nm) and around 4% of middle wave UV-B (280-315 nm), which is known as the most aggressive in DNA damaging. The experimentally UV dose used in this study is approximately 100-fold more effective than UV-B in cyclobutane pyrimidine dimers (CPDs) formation (Kuluncsics *et al.* 1999), which is a measure for irradiation damage. The lowest UV dose used in this study (5 $J/m^2$, **Chapter 2**) significantly induced cellular aggregation, therefore, we assume that UV-induced DNA exchange system reflects the behavior of *Sulfolobus* which it encounters in its natural environment. Thermoacidophilic *Sulfolobus* species thrive under constant stress of a DNA damaging environment through

the accumulation of reactive oxygen species, deamination of nucleotide bases and UV exposure. However, the rate of spontaneous mutations in *S. acidocaldarius* has been found to be very similar to other microorganisms, suggesting a steady mechanism for maintaining the genomic stability in harsh conditions (Grogan *et al.* 2001). *Sulfolobus* cells start to form large aggregates very quickly upon UV light. By clumping in aggregates or through the formation of micro biofilms, the cells achieve a higher resistance to general stress factors. However, the pili-mediated aggregation can also help *Sulfolobus* cells to shield each other from harmful UV light. In this thesis we suggest that UV-induced cells start to aggregate to repair UV-damaged DNA lesions via homologous recombination from undamaged parental cells. If exchange of DNA is mediated by pili is not clear. They could well promote intimate cellular contact whereupon the DNA exchange occurs through a mechanism so far unresolved. Also, the direction of DNA transfer is not clear, as no donor and recipients were identified. Still many questions remain to answer. How exactly does DNA transfer occur, are pili used for transfer, recognition or just for bringing cells in close contact? Recent data suggest that the recognition of *Sulfolobus* species is determined by S-layer glycosylation and glycosylation of the ups-pilin subunits (van Wolferen and Albers, unpublished data). However, the exact molecular mechanism of this specific recognition remains to be determined. These are questions that remain to be answered for the future in order to reveal the molecular mechanisms of the UV-induced DNA exchange system, the species-specificity and the role of this process in the natural environment of Sulfolobales.

# 6

# SAMENVATTING EN CONCLUSIES

## INTRODUCTIE

De Archaea vormen het derde domein van het leven op aarde (Woese *et al.* 1977) en zijn interessant voor onderzoek naar aspecten van evolutie, aanpassing aan extreme groeicondities en stress response mechanismen. Qua vorm en grootte lijken Archaea op Bacteriën, maar een groot aantal genen en mechanismen die betrokken zijn bij het verwerken van informatie, zoals DNA replicatie, transcriptie en translatie, vertonen meer overeenkomsten met Eukarya dan met Bacteria. Daarnaast hebben Archaea ook veel unieke (metabole) eigenschappen die in geen van de beide andere domeinen voorkomen, zoals ongewone routes en enzymen voor koolhydraat metabolisme (Siebers and Schönheit 2005). Veel Archaea leven onder extreme omstandigheden, zoals hete bronnen (hyperthermofielen) of zoutmeren (halofielen), maar met de ontdekking van de mesofiele Archaea werd duidelijk dat ze zich niet beperken tot extreme milieus. Inmiddels zijn er methoden ontwikkeld om Archaea onder laboratoriumcondities te kweken. Voor sommige soorten zijn er zelfs methoden beschikbaar voor het uitschakelen van genen en het gericht tot expressie brengen van eiwitten. Deze ontwikkelingen maken de Archaea een bijzonder interessante groep micro-organismen om te bestuderen.

De *Sulfolobus* soorten behoren tot de best gekarakteriseerde Archaea. Ze behoren tot het thermoacidofiele rijk van de Crenearchaeota en leven meestal in vulkanische bronnen bij een optimale temperatuur van 80°C en een lage, zure pH (pH 2-3). De bekendste stammen van de Sulfolobaceae familie zijn *Sulfolobus acidocaldarius*, het eerste hyperthermofiele organisme dat werd ontdekt in Yellowstone National Park (Brock *et al.* 1972) en *Sulfolobus solfataricus* P2 van de Solfatara velden bij de vulkaan Vesuvius dichtbij Napels in Italië (Zillig *et al.* 1980). Het genoom van *Sulfolobus solfataricus* was het eerste genoom van de Sulfalobales waarvan de sequentie bepaald werd (She *et al.* 2001) en verscheidene andere genomen volgden snel. Omdat *Sulfolobus* stammen redelijk eenvoudig in het laboratorium te kweken zijn, worden ze nu gebruikt als model organisme voor onderzoek aan Crenearchaeota. Het werk dat in dit proefschrift beschreven staat werd gedaan met *S. solfataricus* en *S. acidocaldarius*.

Gedurende de evolutie is de aanpassing aan extreme omstandigheden van essentieel belang geweest voor de ontwikkeling van hyperthermofiele organismen. De overdracht van DNA, met name middels horizontale genoverdracht (HGT), is een belangrijke factor gebleken in de evolutie van het microbiële genoom. Bovendien speelt het uitwisselen van DNA een belangrijke rol in het herstel van DNA via homologe recombinatie, vooral onder extreem thermofiele condities die schadelijk zijn voor het DNA. Voor Archaea en Bacteriën zijn er drie basis mechanismen voor DNA transport gevonden: natuurlijke transformatie, conjugatie

en transductie. Conjugatie is het belangrijkste mechanisme voor horizontale genoverdracht. Het is de eenzijdige overdracht van genetisch materiaal tussen cellen waarbij direct contact of een soort verbindingsbrug tussen de cellen nodig is. **Hoofdstuk 1** van dit proefschrift geeft een overzicht van de verschillende DNA uitwisselingsmechanismen in hyperthermofiele Archaea en Bacteriën. Verder benadrukt dit hoofdstuk de rol van DNA-uitwisseling in het overleven en aanpassen van hyperthermofiele organismen onder extreme condities. De daarop volgende hoofdstukken beschrijven experimentele studies naar een DNA overdrachtmechanisme dat wordt gevonden in Sulfolobales. Dit mechanisme maakt gebruik van zogenaamde type IV pili en wordt geïnduceerd door UV. De complexe transcriptionele reactie op UV-straling die zowel in *S. solfataricus* als S. *acidocaldarius* werd gevonden (Fröls *et al.* 2007; Götz *et al.* 2007) leidde tot de identificatie van de UV-geïnduceerde pili. De vorming van deze pili aan het celoppervlak en het daaropvolgende aggregeren van cellen bleek tot meer DNA-overdracht tussen *Sulfolobus* cellen te leiden. Dit proefschrift beschrijft de eerste waarnemingen van dit UV-geïnduceerde DNA uitwisselingssysteem in hyperthermofiele *Sulfolobus* soorten.



## UV-INDUCEERBARE AGGREGATIE EN TYPE IV PILI VAN SULFOLOBALES

Het pili biogenese operon bevat enkele van de sterkst UV-geïnduceerde genen van Sulfolobales. Dit systeem wordt het *ups*-operon (UV-induceerbare pili van Sulfolobus) genoemd en bestaat uit vijf genen die coderen voor de structurele componenten van een pili opbouw systeem inclusief de pilin onderdelen. Het betreft een ATPase (UpsE), twee pre-pilin onderdelen (UpsA and B), een transmembraan eiwit (UpsF) en een eiwit met onbekende functie (UpsX). Het *ups*-operon bleek essentieel voor de UV-geïnduceerde productie van type IV pili. De pili konden zichtbaar worden gemaakt met behulp van transmissie elektronenmicroscopie aan het oppervlak van UV-geïnduceerde cellen. Bovendien aggregeerden de cellen in clusters van talloze cellen in reactie op behandeling met UV. Verschillende condities werden onderzocht om deze UV-geïnduceerde filamenten en het aggregatieverschijnsel in *S. solfataricus* te karakteriseren (**Hoofdstuk 2**). Het recentelijk ontwikkelde genetische systeem voor de inactivatie van genen (Albers and Driessen) werd gebruikt om de vermeende secretie ATPase UpsE (SSO0120) uit te schakelen. Deze Δ*upsE* stam was niet alleen verstoord in de synthese van pili aan het celoppervlak, maar ook in de UV-geïnduceerde aggregatie van cellen. De pili van *S. solfataricus* bleken rechte filamenten met een variabele lengte te zijn met een uniforme diameter van ongeveer 10 nm. Ze zijn duidelijk anders en talrijker dan de archaella (voorheen flagella genoemd) van *S. solfataricus*.

De pilus filamenten verschenen aan het cel oppervlak in reactie op UV-stress na ongeveer één uur UV-straling. Tot 80% van de cellen in de cultuur vormden zeer dynamische cel aggregaten. De maximale aggregatie werd 6-8 uur na UV-behandeling bereikt, gevolgd door langzame disaggregatie. Het aantal aggregaten bleek afhankelijk van de UV-dosis en de meeste cellen werden gedood bij hoge UV-doses (200 or 1000 J/m$^2$). Opmerkelijk was dat de cel aggregatie ook kon worden geïnduceerd door stoffen die de dubbele streng van het DNA verbreken, zoals bleomycin en mitomycin C. Andere stress factoren, zoals veranderingen in pH of temperatuur resulteerden echter niet in cel aggregatie. Deze resultaten suggereren dat aggregatie en pili vorming met elkaar verbonden processen zijn die misschien het herstel van het chromosoom van de aggregerende cellen met behulp van recombinatie mogelijk maken. Het gedetailleerde mechanisme van de UV-geïnduceerde aggregatie en de structurele kenmerken van de cellen in deze aggregaten zijn echter nog niet bekend.

Het onderzoek naar het UV-induceerbare type IV pilus system werd voortgezet met de genetisch meer toegankelijke soort *S. acidocaldarius* (**Hoofdstuk 3**). Tegenwoordig is *S. acidocaldarius* genetisch meer toegankelijk omdat deletie mutanten eenvoudig te creëren zijn door middel van dubbele cross-over recombinatie (Wagner *et al.* 2012; Wagner *et al.* 2009). Om meer inzicht te krijgen in de specificiteit van de cel-cel herkenning tussen UV-geïnduceerde *Sulfolobus* cellen werd de aggregatie geanalyseerd met behulp van fluorescente *in situ* hybridisatie. Er werd een duidelijk soortafhankelijk herkenningsproces waargenomen. De UV-geïnduceerde pili van verschillende *Sulfolobus* stammen werden onderzocht en de filamenten van *S. solfataricus*, *S. acidocaldarius* en *S. tokodaii* bleken in lengte te verschillen. *S. acidocaldarius* produceerde de kortste en meest talrijke ups-pili. Het is waarschijnlijk dat de verschillen in pilus morfologie tussen de *Sulfolobus* stammen het resultaat is van de verschillende aminozuur sequenties van de individuele pili eenheden.

## UV-INDUCEERBARE DNA UITWISSELING IN SULFOLOBALES

De aggregatie van cellen in de thermoacidofiele crenarchaeoot *Sulfolobus* sp. werd al in een vroeg stadium met DNA overdracht door middel van conjugatie in verband gebracht (Schleper *et al.* 1995). Bovendien was aangetoond dat UV behandeling de uitwisseling van chromosomale merkers in *S. acidocaldarius* deed toenemen (Schmidt K *et al.* 1999). De mechanismen en genen die voor deze DNA overdracht verantwoordelijk waren bleven echter onbekend. De thermoacidofiele *Sulfolobus* sp. voelt zich thuis in een omgeving met continue stress en DNA beschadigende condities met mogelijk hoge UV-doses. Onder dit soort condities zijn mogelijk speciale DNA reparatie mechanismen nodig om de levensvatbaarheid van de cellen

te waarborgen. Microarray analyse van de UV-specifieke transcriptie reactie in *Sulfolobus* sp. leidde niet tot identificatie van genen die betrokken waren bij direct DNA herstel (Fröls *et al.* 2007; Götz *et al.* 2007). **Hoofdstuk 3** van dit proefschrift beschrijft een uniek DNA uitwisselingsmechanisme dat werd waargenomen tijden UV-geïnduceerde cel aggregatie, een proces dat afhankelijk is van type IV-pili. Het onderzoek dat in **Hoofdstuk 2** beschreven staat had tot de vraag geleid wat de functie van de door UV-geïnduceerde pili bemiddelde aggregatie was. Om de rol van dit proces the onderzoeken werden kruisingsexperimenten uitgevoerd (Hansen *et al.* 2005). Cel aggregatie bleek de uitwisseling van chromosomale merkers te bevorderen tot op recombinatie frequenties die tot drie orders van grootte hoger lagen dan die van cellen die niet met UV behandeld waren. In de kruisingsexperimenten werden ook stammen onderzocht waarin de pili genen verwijderd waren en die daardoor gestoord waren in de productie van pili en de aggregatie van cellen. De overlevingskans van deze stammen na blootstelling aan UV bleek lager dan in de oorspronkelijke stam. Wanneer de stammen waarin de pili genen verwijderd waren (Δ*upsEF*) werden gekruist met een wild-type partner die bestraald was met UV, leverde dit nog steeds recombinanten op. Maar wanneer beide kruisingspartners pili misten werd er geen uitwisseling van DNA gevonden. Tezamen tonen deze data een nieuwe rol voor type IV pili aan, waarin de pili cel aggregatie bevorderen en daarbij DNA overdracht toestaan met als doel het herstel van DNA dat beschadigd is door UV (Figuur 1).

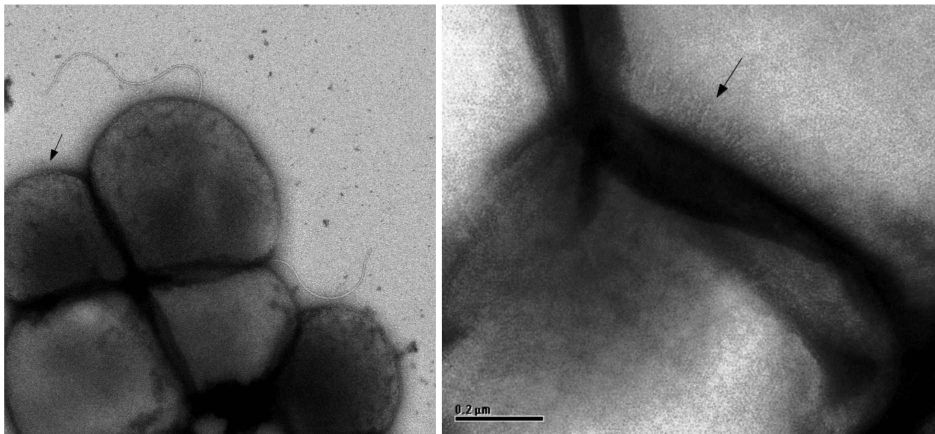



**Figure 1.** Elektronenmicroscopische beelden van wild type *Sulfolobus acidocaldarius* cellen die onder invloed van UV clusteren. (A) Ups-pili die gevormd zijn aan het oppervlak van UV-geïnduceerde cel aggregaten 3 uur na UV behandeling. (B) UV-geïnduceerde pili werden ook tussen de cellen in de aggregaten waargenomen. De pili zijn aangegeven met pijlen.

## MOLECULAIRE ANALYSE VAN HET *UPS*-OPERON VAN *S. ACIDOCALDARIUS*

In **Hoofdstuk 2** en **3** was al aangetoond dat de secretie ATPase (UpsE) en het transmembraaneiwit (UpsF) nodig zijn voor de biogenese van ups-pili, cel aggregatie en DNA uitwisseling in UV bestraalde *Sulfolobus* cellen. De resterende genen van het *Sulfolobus ups*-operon (UpsX, UpsA and UpsB) werden in Hoofdstuk 4 van dit proefschrift geanalyseerd met behulp van bioinformatica, transcriptie analyse, gen deletie en lokalisatie experimenten. Speciale aandacht werd besteed aan het karakteriseren en definiëren van de rol van UpsX, een eiwit met onbekenden functie dat geen geconserveerde domeinen bevat. UpsX is echter een zeer geconserveerd eiwit in alle *Sulfolobus* soorten. De transcriptie niveaus van de *ups* genen werden in alle deletie stammen geanalyseerd. Al deze stammen (Δ*upsE*, *F*, *A* and *B*), behalve Δ*upsX* stam, waren gestoord in cel aggregatie onder invloed van UV. Bovendien, en zoals verwacht, werden er geen ups-pili opgebouwd in de Δ*upsE* en Δ*upsF* cellen, omdat de ATPase en het membraaneiwit essentieel zijn voor de opbouw van type IV pili. Het was interessant om te zien dat de mutanten waarin slechts één van de pili onderdelen was uitgeschakeld nog steeds pili konden vormen, maar vergeleken met de wild-type werden deze pili in zeer kleine hoeveelheden gemaakt. Waarschijnlijk maken beide pili eiwitten onderdeel uit van de ups-pili. Opvallend genoeg liet de *upsX* deletie stam een ander fenotype zien. In tegenstelling tot de andere genen van het *ups*-operon vervult UpsX geen essentiële rol in de opbouw van pili of cel aggregaten en de mutant heeft nog steeds dezelfde eigenschappen als de wild-type in deze processen. Desalniettemin kan een mutant die zowel het *upsX* gen en zijn promoter mist niet langer pili filamenten maken. Diepe sequentie analyse van *S. acidocaldarius* cDNA liet niet alleen een transcriptie start punt (TSS) voor het *upsX* gen zien, maar ook extra TSSs voor *upsE* en *upsA*. Dit suggereert dat deze secondaire transcriptie start gebruikt kan worden om de stoichiometrie van de pilin eiwitten in de ups-pili te optimaliseren. Om de rol van UpsX in DNA uitwisseling tussen *Sulfolobus* cellen te analyseren werd het experiment van **Hoofdstuk 3** gebruikt. In een conjugatie experiment waarin de wild-type stam werd gemengd met UV-geïnduceerde Δ*upsX,* was het aantal recombinanten hoger dan zonder UV-bestraling, maar ongeveer 50% van het aantal dat in een kruisingsexperiment met alleen wild-type stammen werd waargenomen. Dit suggereert dat UpsX een rol speelt in het behouden van efficiënte DNA overdracht tussen *Sulfolobus* cellen, maar dat deze rol niet essentieel is. Onder invloed van UV bevindt zich een groot deel van de UpsX uit de cel zich aan de membranen, terwijl UpsX voorspeld is een cytoplasmatisch eiwit te zijn. Mogelijk is UpsX betrokken bij het DNA overdrachtsmechanisme hoewel op dit moment nog niet bekend is wat het doet tijdens UV-geïnduceerde conjugatie.

## SAMENVATTENDE OPMERKINGEN EN TOEKOMSTIG ONDERZOEK

Type IV pili zijn filamentachtige structuren die betrokken zijn bij verscheidene functies in veel Bacteriën en Archaea. De functies die aan pili worden toegekend bestaan vooral uit de beweeglijkheid van cellen, adhesie aan oppervlakken, het vormen van biofilms en de opname van DNA. In dit proefschrift werd gevonden dat type IV pili een rol spelen in UV-geïnduceerde uitwisseling van chromosomale DNA en cel aggregatie in hyperthermofiele *Sulfolobus* soorten. De pili konden worden geïnduceerd onder laboratorium condities met hoge energie korte golf UV-C licht (100-280 nm) met een golflengte van 254 nm. In de natuur bestaat zonlicht uit ongeveer 96% lange golf UV-A (315-400 nm) en ongeveer 4% middellange golf UV-B (280-315 nm), die bekend staat als het meest agressief in het beschadigen van DNA. De experimentele UV-dosis die in deze studie werd gebruikt is ongeveer 100 keer effectiever dan UV-B in het vormen van cyclobutane pyrimidine dimers (CPDs) (Kuluncsics *et al.* 1999), wat een maat is voor stralingsschade. De laagste UV dosis die gebruikt werd in deze studie (5 $J/m^2$, **Hoofdstuk 2**) induceerde significante cel aggregatie en daarom gaan we ervan uit dat UV-geïnduceerde DNA uitwisseling het gedrag *Sulfolobus* in zijn natuurlijke omgeving reflecteert. Thermoacidofiele *Sulfolobus* soorten gedijen goed onder de constante stress van DNA beschadigende condities zoals de aanwezigheid van reactieve zuurstofdeeltjes, deaminatie van nucleotide basen en blootstelling aan UV. Desalniettemin blijkt de snelheid waarmee spontane mutaties in *S. acidocaldarius* plaatsvinden bijna gelijk te zijn aan die van andere micro-organismen, wat suggereert dan er een vast systeem is voor het behouden van de genomische stabiliteit onder extreme omstandigheden (Grogan *et al.* 2001). Onder invloed van UV beginnen *Sulfolobus* cellen zeer snel grote aggregaten te vormen. Door samen te klonteren in aggregaten of door het vormen van biofilms bereiken de cellen een grotere resistentie tegen algemene stress factoren. De door pili bemiddelde aggregatie kan *Sulfolobus* cellen echter ook helpen om elkaar te beschermen tegen schadelijk UV-licht. In dit proefschrift stellen we voor dat UV-geïnduceerde cellen beginnen te aggregeren om het door UV beschadigde DNA via homologe recombinatie met het chromosomale DNA van onbeschadigde cellen te repareren. Het is nog niet duidelijk of de uitwisseling van het DNA door pili bemiddeld wordt. Het is goed mogelijk dat ze slechts het intieme contact tussen cellen stimuleren waarna DNA uitwisseling plaatsvindt via een tot nu toe nog onbekend mechanisme. Bovendien is de richting van de DNA overdracht nog niet bekend, daar er geen donor en ontvangende cellen zijn geïdentificeerd. Er blijven nog steeds veel vragen over. Hoe vindt de DNA overdracht precies plaats, worden de pili gebruikt voor overdracht, herkenning of alleen om cellen met elkaar in contact te brengen? Recente gegevens suggereren

dat de herkenning van *Sulfolobus* species wordt bepaald door de mate van suiker modificatie van de S-laag en de ups-pilin onderdelen (van Wolferen and Albers, niet gepubliceerde data). Desondanks moet het exacte moleculaire mechanisme van deze specifieke herkenning nog bepaald worden. Dit zijn vragen die in de toekomst beantwoord moeten worden om het moleculaire mechanisme van het UV-geïnduceerde DNA uitwisselingssysteem, de soort specificiteit en de rol van dit proces in de natuurlijke omgeving van Sulfolobales op te helderen.

# 7

## PODSUMOWANIE

## WPROWADZENIE

Archeony (*Archaea*), zwane uprzednio archeobakteriami lub archeowcami, stanowią jeden z najbardziej interesujących obiektów badawczych współczesnej mikrobiologii. Organizmy te charakteryzują się wyjątkową odpornością i zdolnością do życia w środowisku o ekstremalnie wysokich temperaturach, dużym zasoleniu oraz bardzo niskim, kwaśnym pH. Archeony zamieszkują obszary niedostępne dla innych form życia, takie jak wulkany, lodowce czy gejzery. Przedstawiciele *Archaea* znajdywani są również w jeziorach solnych, na dnie oceanów, a także w przewodach pokarmowych organizmów wyższych, np. człowieka.

Ze względu na podobieństwo morfologiczne do bakterii, archeony przez wiele lat zaliczane były do nadkrólestwa (domeny) bakterii. Dopiero w 1977 roku Carl Woese - poprzez analizę sekwencji nukleotydowej rybosomalnego RNA - przedefiniował stary system klasyfikacji prokaryota i eukaryota. Analiza filogenetyczna sklasyfikowała archeony jako osobną, trzecią domenę życia. Pomimo ich fizycznego podobieństwa do bakteri wiele genów i procesów odpowiedzialnych za przekaz informacji (replikacja DNA, transkrypcja i translacja) archeonów jest podobna do tych u organizmów jądrowych, eukaryota. Ponadto wiele genów archeonów jest unikalnych i nie ma odpowiedników w genomach bakterii czy eukaryota.

Jednym z najlepiej poznanych archeonów jest rodzina Sulfolobaceae. Należące do niej rodzaje *Sulfolobus* są przedstawicielami królestwa Crenearchaeota. *Sulfolobus* zamieszkują głównie gorące źródła wulkaniczne, gdzie temperatura sięga nawet 80°C, a pH jest kwaśne (pH 2-3). Najlepiej zbadanymi szczepami z rodziny Sulfolobaceae są *Sulfolobus acidocaldarius*, pierwszy hipertermofil odkryty w Parku Narodowym Yellowstone (Brock *et al.* 1972) oraz *Sulfolobus solfataricus* P2 z solfatar wulkanu Wezuwiusz w okolicach Neapolu we Włoszech (Zillig *et al.* 1980). Genom *Sulfolobus solfataricus* był pierwszym z sekwencjonowanych genomów spośród rzędu Sulfolobales (She *et al.* 2001). Jako że szczepy *Sulfolobus* nie są zbyt wymagające w hodowli laboratoryjnej, organizm ten stał się organizmem modelowym królestwa Crenearchaeota, a szczepy *S. solfataricus* i *S. acidocaldarius* wykorzystano w badaniach przedstawionych w niniejszej pracy.

W czasie ewolucji dostosowanie się do warunków ekstremalnych było kluczowe dla organizmów hipertermofilnych. Transfer DNA, szczególnie horyzontalny (poziomy) transfer genów, to niezwykle istotny czynnik w ewolucji genomów wszystkich mikroorganizmów. Ponadto wymiana materiału genetycznego, niezwykle istotna w warunkach sprzyjających uszkodzeniom DNA, odgrywa istotną rolę w procesach naprawy DNA poprzez homologiczną rekombinację. Dla archeonów i bakterii zostały scharakteryzowane trzy podstawowe mechanizmy transferu genów - transformacja, koniugacja oraz transdukcja. Główny mechanizm zaangażowany w poziomy transfer genów to koniugacja, czyli jednokierunkowy

transport materiału genetycznego, w którym bezpośredni kontakt między komórkami jest bezwzględnie wymagany. W **Rozdziale 1** niniejszej pracy omówiono różne mechanizmy wymiany DNA w hipertermofilnych archeonach oraz bakteriach. Został ponadto podkreślony udział procesu wymiany genów w adaptacji hipertermofilnych organizmów do życia w środowisku ekstremalnym. Kolejne rozdziały opisują prace badawcze dotyczące mechanizmu transferu genów u Sulfolobales z udziałem pilusów typu IV po indukcji światłem ultrafioletowym. Złożona odpowiedź transkrypcyjna *Sulfolobus solfataricus* oraz *S. acidocaldarius* w wyniku naświetlania promieniowaniem UV doprowadziła do identyfikacji nowego typu pilusów. Pilusy te oraz powstające pod wpływem światła UV skupiska komórkowe (agregacje) stymulują wymianę materiału genetycznego pomiędzy komórkami szczepów *Sulfolobus*. W niniejszej pracy przedstawiono pierwsze badania nad indukowanym światłem UV systemem wymiany materiału genetycznego pomiędzy hipertermofilnymi szczepami *Sulfolobus*.



## AGREGACJE INDUKOWANE ŚWIATŁEM UV ORAZ PILUS TYPU IV U SULFOLOBALES

Operon odpowiedzialny za biogenezę pilusa gromadzi geny najsilniej indukowane światłem UV; jest nazywany operonem *ups* od pierwszych liter angielskiego 'UV-inducible pili operon of *Sulfolobus*', czyli UV-indukowanego operonu pilusa w szczepie *Sulfolobus*. Operon ten składa się z pięciu genów kodujących elementy konstrukcyjne budujące trzon pilusa oraz z podjednostek białek strukturalnych (pilin). Są to ATPaza (UpsE), dwie pre-piliny (UpsA i B), białko transbłonowe (UpsF) oraz białko o nieznanej funkcji (UpsX). W toku przedstawionych tu badań udowodniono, że *ups*-operon jest odpowiedzialny za powstawanie pilusa typu IV, zależnego od światła UV.

Pilusy powstałe na powierzchni komórek *S. solfataricus* po napromieniowaniu światłem UV obserwowano za pomocą transmisyjnego mikroskopu elektronowego. Co ciekawe, oprócz wytwarzania pilusów naświetlane komórki tworzyły liczne skupiska (agregacje). Fenomen ten, powstawanie filamentów oraz agregacji komórkowych, tworzących się w wyniku promieniowania ultrafioletowego, zostały zbadane w rozmaitych warunkach (**Rozdział 2**). Pilusy *S. solfataricus* to proste filamenty o różnej długości, ale zbliżonej średnicy - około 10 nm. Wizualnie różne od powszechnie występującej u *Sulfolobus* wici archaellum (zwanej uprzednio flagellum). Pilus powstaje na powierzchni komórki w odpowiedzi na stres wywołany promieniowaniem UV już około godziny po naświetleniu. Ponadto w ciągu pierwszych 6-8 godzin po naświetleniu ponad 80% komórek formuje się w skupiska (agregacje). Potem ilość skupisk stopniowo ulega zmniejszeniu. Ilość komórek w agregacjach zależy od dawki użytego UV, a większość komórek ulega

zniszczeniu przy zbyt wysokich dawkach UV (200 bądź 1000 $J/m^2$). Co ciekawe, komórki *Sulfolobus* agregują również pod wpływem związków uszkadzających strukturę nici DNA, takich jak bleomycyna i mitocyna C. Jednakże inne czynniki stresogenne, jak zmiany pH czy wahania temperatury, nie powodowały komórkowych agregacji.

Z kolei niedawno opracowane techniki inżynierii genetycznej u *Sulfolobus solfataricus* (Albers i Driessen 2008) umożliwiły inaktywację genu sekrecyjnej ATPazy *upsE* (*sso0120*). Szczep z delecją genu *upsE* był niezdolny do produkcji pilusa na swojej powierzchni, a naświetlone UV komórki nie tworzyły agregacji.

Wyniki powyższych badań sugerują wzajemne powiązanie procesu agregacji oraz tworzenia pilusa, prawdopodobnie w celu naprawy chromosomalnego DNA agregujących komórek. Jednakże szczegółowy mechanizm agregacji wywoływanej światłem UV oraz strukturalne cechy komórek w agregacjach wciąż pozostają nieznane.

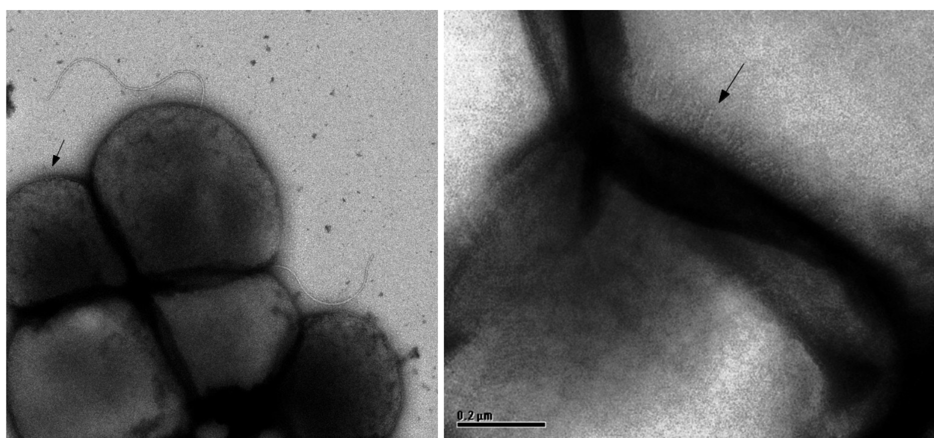
Dalsze badania nad systemem indukowanego UV pilusa typu IV kontynuowano na szczepie *Sulfolobus acidocaldarius*, który jest organizmem bardziej stabilnym genetycznie niż *S. solfataricus* (**Rozdział 3**). Tworzenie mutantów delecyjnych u *S. acidocaldarius* bazuje obecnie na procesie rekombinacji homologicznej i krzyżowej wymianie fragmentów nici (ang. *crossing-over*) (Wagner *et al.* 2012; Wagner *et al.* 2009). Aby określić swoistość agregujących pod wpływem światła UV komórek, zastosowano metodę fluorescencyjnej hybrydyzacji *in situ*. Zaobserwowano jednoznaczną specyficzność gatunkową w rozpoznawaniu agregujących ze sobą komórek. Ponadto analizowano ups-pilusy w różnych szczepach *Sulfolobusu*. Filament ma różną długość w *S. solfataricus*, *S. acidocaldarius* i *S. tokodaii*, a najdłuższe i najliczniejsze ups-pilusy powstają u *S. acidocaldarius*. Za różnice w morfologii pilusów prawdopodobnie odpowiadają różnice w sekwencji aminokwasowej poszczególnych białek strukturalnych (pilin) w homologicznych szczepach *Sulfolobus*.

## INDUKOWANA PROMIENIOWANIEM UV WYMIANA DNA U SULFOLOBALES

Komórkowe agregacje hipertermofilnych szczepów *Sulfolobus* były już łączone z koniugacyjnym transferem DNA we wcześniejszych badaniach (Schleper *et al.* 1995). Pokazano ponadto, że stymulacja światłem UV zwiększa wymianę chromosomalnych markerów DNA u *S. acidocaldarius* (Schmidt *et al.* 1999). Jednakże mechanizm oraz geny odpowiedzialne za transfer DNA wciąż pozostawał nieznany. Termoacidofilne szczepy *Sulfolobus* żyją w warunkach ciągłego stresu, w środowisku z potencjalnie wysokim natężeniem szkodliwego dla DNA światła UV. By komórki mogły funkcjonować w tak niekorzystnych warunkach, niezbędne są specjalne mechanizmy naprawcze DNA. Jednak analiza mikromacierzy DNA

*Sulfolobus* po napromieniowaniu światłem UV nie wykazała genów zaangażowanych bezpośrednio w naprawę DNA (Fröls *et al.* 2007; Götz *et al.* 2007). **Rozdział 3** niniejszej pracy przedstawia unikalny proces wymiany DNA, który zachodzi podczas indukowanych światłem UV agregacji komórkowych i zależny jest od pilusa typu IV. Badania opisane w **Rodziale 2** postawiły istotne pytanie o funkcje agregacji wywołanych UV. W celu zbadania ich znaczenia szczepy *Sulfolobus* łączono w pary w eksperymencie koniugacji (ang. *mating experiment*) (Hansen *et al.* 2005). Pokazano, że powstawanie agregacji między komórkami stymuluje wymianę markerów chromosomalnych z częstotliwością rekombinacji do trzech rzędów wielkości wyższą niż w przypadku komórek nienaświetlanych UV (nieagregujących). W eksperymencie badano również szczepy delecyjne pozbawione genów pilusa, czyli niezdolne do syntezy pilusa oraz nie tworzące agregacji. Warto podkreślić, że szczepy te po naświetleniu UV wykazywały obniżoną przeżywalność w stosunku do szczepów dzikich (rodzicielskich), tworzących pili i agregujących. Szczepy niezdolne do syntezy pili (Δ*upsEF*) wciąż tworzyły rekombinanty po złączeniu z partnerskim dzikim szczepem, naświetlonym światłem UV. Jednak gdy oba szczepy partnerskie były niezdolne do utworzenia pilusa, wymiana markerów DNA była niemożliwa. Podsumowując, wyniki tych badań przedstawiają zatem nową rolę pilusa typu IV, który pośredniczy w komórkowych agregacjach pozwalając na wymianę materiału genetycznego, a w związku z tym naprawę DNA zniszczonego działaniem UV (Rysunek 1).






**Rysunek 1.** Zdjęcie z mikroskopu elektronowego agregacji komórkowych dzikiego szczepu *Sulfolobus acidocaldarius* po naświetleniu UV. (A) Ups-pilusy powstające na powierzchni agregujących komórek 3 godziny po naświetleniu światłem UV. (B) Indukowane światłem UV pilusy zaobserwowano również pomiędzy komórkami w agregatach. Centra pilusów oznaczono strzałkami.

## ANALIZA MOLEKULARNA *UPS*-OPERONU U *S. ACIDOCALDARIUS*

Sekrecyjna ATPase (UpsE) oraz białko transmembranowe (UpsF) z *ups*-operonu są niezbędne w biogenezie ups-pili, w komórkowych agregacjach oraz w procesie wymiany DNA pomiędzy naświetlonymi UV komórkami *Sulfolobus* (**Rozdział 2** oraz **Rozdział 3**). Pozostałe geny *ups*-operonu (*upsX*, *upsA* i *upsB*) analizowano w **Rozdziale 4** za pomocą bioinformatyki, analizy transkrypcyjnej, delecji genów oraz poprzez eksperymenty określające lokalizację białek w komórce. Szczególnie skupiono się na charakterystyce oraz nakreśleniu roli UpsX, białka o nieznanej funkcji oraz pozbawionego charakterystycznych konserwatywnych domen. UpsX jest białkiem występującym jedynie u szczepów *Sulfolobus*. Poziom transkrypcji genów *ups* został zbadany we wszystkich mutantach delecyjnych. Wszystkie szczepy delecyjne (Δ*upsE*, *F*, *A* oraz *B*) były niezdolne do tworzenia agregacji komórkowych pod wpływem światła UV. Jedynym wyjątkiem był wciąż agregujący szczep Δ*upsX*. Zgodnie z wcześniejszymi oczekiwaniami szczepy z delecją genów *upsE* i *upsF* nie produkowały ups-pilusa, jako że ATPaza oraz białko błonowe są niezbędne do biogenezy pilusa typu IV. Co ciekawe, pojedyncze mutanty pozbawione jednej z podjednostek strukturalnych pilusa- piliny UpsA czy UpsB wciąż wytwarzały filament na powierzchni komórek, aczkolwiek liczba pilusów była dużo mniejsza niż w komórkach szczepu dzikiego. Prawdopodobnie więc obie piliny są równoważne w budowaniu struktury ups-pilusa. Wyjątkowo szczep delecyjny Δ*upsX* miał fenotyp inny od reszty mutantów z *ups*-operonu. W przeciwieństwie do pozostałych genów operonu UpsX nie pełni istotnej roli w kształtowaniu pilusa czy tworzeniu komórkowych agregacji, szczep Δ*upsX* wciąż agreguje oraz wytwarza pilusa typu IV pod wpływem promieni UV. Jednak mutant pozbawiony genu *upsX* oraz obszaru jego promotora jest już niezdolny do wytworzenia filamentu (pilusa) ups. Głębokie sekwencjonowanie cDNA szczepu *Sulfolobus acidocaldarius* ujawniło występowanie miejsca startu transkrypcji nie tylko przed genem *upsX*, ale także dodatkowo przed genami *upsE* oraz *upsA*. Można by spekulować, że istnienie dodatkowego miejsca rozpoczęcia transkrypcji pozwala na bardziej precyzyjne dopasowanie stechiometrii pilin budujących strukturę ups-pilusa. By poznać rolę UpsX w procesie wymiany materiału genetycznego pomiędzy komórkami *Sulfolobus*, użyto testu przedstawionego w Rozdziale 3. Przeprowadzając eksperyment koniugacji, mieszając ze sobą szczep dziki i Δ*upsX*, indukowany UV, zaobserwowano, że ilość powstających rekombinantów jest wyższa niż w przypadku komórek nienaświetlanych ultrafioletem, jednak nie przekracza 50% ilości rekombinantów, powstających przy łączeniu tylko komórek szczepu dzikiego. Wynik ten sugeruje, że białko UpsX odgrywa rolę w utrzymaniu sprawnego transferu DNA pomiędzy komórkami *Sulfolobus*, jednak

nie jest niezbędne do zajścia wymiany genów. Ponadto po naświetleniu UV duża część białka UpsX umiejscawia się w błonach komórkowych, pomimo że UpsX jest potencjalnym białkiem cytozolowym. UpsX wydaje się zatem być zaangażowany w proces transferu DNA, jednak obecnie dokładny mechanizm udziału tego białka w indukowanej promieniowaniem UV koniugacji nie jest znany.

## UWAGI KOŃCOWE

Pilusy typu IV to nitkowate struktury biorące udział w różnorodnych procesach komórkowych w bakteriach oraz w archeonach. Zaangażowane są przede wszysktim w ruch komórek oraz przyczepianie do podłoża stałego, biorą też udział w tworzeniu biofilmu oraz w pobieraniu obcego DNA ze środkowiska. W niniejszej pracy pokazano udział pilusów typu IV w indukowanej światłem UV wymianie DNA (chromosomalnych markerów) oraz w agregacji komórek hipertermofilnego szczepu *Sulfolobus*. Pilusy w warunkach laboratoryjnych powstają pod wpływem promieniowania ultrafioletowego UV-C (100-280 nm) o długości fali 254 nm. W naturze słońce emituje około 96% promieniowania ultrafioletowego w zakresie UV-A (315-400 nm) i tylko około 4% w zakresie UV-B (280-315 nm), które to promieniowanie jest najbardziej toksyczne dla struktury DNA. Dawka UV użyta w powyższych badaniach jet około 100 razy bardziej skuteczna w tworzeniu cyklobutanowych dimerów pirymidynowych (CPD) od UV-B (Kuluncsics *et al.* 1999), co stanowi miarę popromiennych uszkodzeń DNA. Najniższa dawka UV użyta w powyższej pracy (5 J/m$^2$, **Rozdział 2**) była wystarczająca do indukcji agregacji komórkowych, stąd też wnioskować można, że odkryty system wymiany DNA pod wpływem promieniowania UV odzwierciedla zachowanie komórek *Sulfolobus* w środowisku naturalnym. Termoacidofilne szczepy *Sulfolobus* bytują w warunkach ciągłego stresu, w środowisku szkodliwym dla DNA na skutek nagromadzenia się reaktywnych form tlenu, deaminacji nukleotydowej oraz promieniowania UV. Niemniej jednak stwierdzono, że tempo powstawania spontanicznych mutacji u *S. acidocaldarius* nie odbiega od wartości wyznaczonych dla innych mikroorganizmów, co sugeruje występowanie stałego mechanizmu utrzymania stabilności genomu u *Sulfolobus* (Grogan *et al.* 2001). Komórki *Sulfolobus* zaczynają tworzenie dużych agregatów bardzo szybko po naświetleniu UV. Komórki osiągają większą odporność na ogólne czynniki stresowe poprzez zlepianie się w agregaty czy przez formacje mikro biofilmów. Agregacje za pośrednictwem pilusa mogą również pomóc komórkom *Sulfolobus* w ochronie przed szkodliwym promeniowaniem UV. W oparciu o wyniki zgromadzone w niniejszej pracy wnioskujemy, że komórki napromieniowane światłem UV tworzą agregacje, by naprawić uszkodzone działaniem UV DNA poprzez homologiczną rekombinację fragmentów DNA z nieuszkodzonych

komórek rodzicielskich. To, czy pilus bezpośrednio pośredniczy w transporcie DNA, pozostaje niejasne. Prawdopodobnie pilusy promują intymne kontakty międzykomórkowe, podczas gdy wymiana DNA przebiega w sposób dotychczas nieznany. Mimo znacznego postępu w badaniach nadal wiele pytań pozostaje bez odpowiedzi. Jaki jest kierunek przenoszenia DNA? Ani dawca, ani biorca nie zostali bowiem dotychczas zidentyfikowani. Jak dokładnie wygląda transport DNA? Czy pilus jest niezbędny w procesie wymiany DNA przy rozpoznawaniu komórek, czy tylko do zapewnienia bliskiego kontaktu komórkom? Najnowsze dane wskazują, że specyficzne rozpoznawanie szczepów *Sulfolobus* jest zdeterminowane unikalną glikozylacją warstwy S (ang. *S-layer*) oraz samych podjednostek pilin ups (van Wolferen oraz Albers, dane niepublikowane). Jednakże dokładny molekularny mechanizm procesu rozponawania komórek pozostaje do ustalenia. Są to pytania, na które należy odpowiedzieć w przyszłości w celu ujawnienia podstaw molekularnych systemu wymiany DNA pod wpływem promieniowania UV, jego specyficzności oraz roli w środowisku naturalnym rzędu Sulfolobales.

# APPENDIX

Supplementary material

Reference list

Acknowledgements

List of publications

# SUPPLEMENTARY MATERIAL

## TABLES

**Table S1 (all chapters).** Primers and probes used in this study.

| Primer | Sequence | Purpose |
|---|---|---|
| 19 | CCCGCGGATCCCGAGATAGGCAATAGTAATATGG | Δ*upsX* -40_US_RV_BamHI |
| 20 | CGCGCCGCGGCATTTAATCCTGCATAGCCTGAG | Δ*upsX* -40_US_FW_SacII |
| 21 | GCGGCCCGGGCTTATGGCATGGATCAGGTATTAG | Δ*upsX* -40_DS_FW_SmaI |
| 22 | GCCCTGCAGCCGTGAAATGTGGTGATGGAAC | Δ*upsX* -40_DS_RV_PstI |
| 546 | CGCGCTGCAGCCTGCATAGCCTGAGCCTACTATAATCG | Δ*upsX*_US_FW_ApaI |
| 547 | GCTAATACCTGATCCATTTATTTCTTCGATGCTGTAAAATATAC | Δ*upsX*_US_RW |
| 548 | CAGCATCGAAGAAATAAATGGATCAGGTATTAGCAGAG | Δ*upsX*_DS_FW |
| 549 | CGCGCGGATCCGTGAAATGTGGTGATGGAACCGTG | Δ*upsX*_DS_RV_BamHI |
| 2000 | GTAGGGCCCCCAGTTAGTTAAGCTTTTACCAG | Δ*upsA*_US_FW_ApaI |
| 2001 | GAGATTCCTTTCATCTTTCCTCAAATAAAATGAATC | Δ*upsA*_US_RW |
| 2002 | TTGAGGAAAGATGAAAGGAATCTCTTCAATTTTTTC | Δ*upsA*_DS_FW |
| 2003 | GCGGATCCGCAATCAAAGCCGACTTGTCTG | Δ*upsA*_DS_RV_BamHI |
| 2004 | GAGGGCCCTCTATTTCCCAACAATTCTAATG | Δ*upsB*_US_FW_ApaI |
| 2005 | GAATAGAATAGTCCTAATTACACCGTAGAAGCTAG | Δ*upsB*_US_RW |
| 2006 | CGGTGTAATTAGGACTATTCTATTCTTTTTTAG | Δ*upsB*_DS_FW |
| 2007 | GCGGATCCAAACCACATCAGCTGTCTTATCAC | Δ*upsB*_DS_RV_BamHI |
| 2008 | GAATAGAATAGTTTTAACCAACTTTCCTCAAATAAAATG | Δ*upsA*B_US_RW |
| 2009 | GAAAGTTGGTTAAAACTATTCTATTCTTTTTTAG | Δ*upsA*B_DS_FW |
| 2010 | GTAGGGCCCGTGTATAATGATGACCTATTTAGCTG | Δ*upsE*_US_FW_ApaI |
| 2011 | CTAATATTTTCAAGCCATAAGAAGGAAATATTAAAAG | Δ*upsE*_US_RV |
| 2012 | CTTCTTATGGCTTGAAAATATTAGCATGTGATATATTC | Δ*upsE*_DS_FW |
| 2013 | GTCGGATCCCTTAATCTATCCTTAAGCGAAACGC | Δ*upsE*_DS_RW_BamHI |
| 2014 | AAGGGATAATAGAGTAGAAC | Δ*upsE*_Check_FW |

**Table S1 (all chapters).** Primers and probes used in this study. (*Continued*)

| Primer | Sequence | Purpose |
|---|---|---|
| 2015 | GTAAACTGGAAGCCTATAAGG | Δ*upsE*_Check_RV |
| 2016 | GTAGGGCCCGATAATAGGTGAGGTAAGAGG | Δ*upsF*_US_FW_ ApaI |
| 2017 | AAATGAATCTTTTAATCACATGCTAATATTTTC | Δ*upsF*_US_RV |
| 2018 | AGCATGTGATTAAAAGATTCATTTTATTTG | Δ*upsF*_DS_FW |
| 2019 | GTCGGATCCGAGATTCCTTTCATCCTAATTAC | Δ*upsF*_DS_RW_ BamHI |
| 2020 | GGTTAGATTTATAGCAAGATCAAC | Δ*upsF*_Check_FW |
| 2021 | GTATTCATAAGAGTTGGATAGCG | Δ*upsF*_Check_RV |
| 2028 | CTTCTCCTAAATGTTAATCTG | Δ*upsA*_Check_FW |
| 2029 | GTTGGATAGCGAATTGGTCG | Δ*upsA*_Check_RV |
| 2030 | ACTTCAAAGATGATTGCAGGAG | Δ*upsB*_Check_FW |
| 2031 | TTTCCACATATGTACGATGAG | Δ*upsB*_Check_RV |
| 2073 | AATTTAGCATAGACCCAGCTTAC | qPCR *upsX* fw |
| 2074 | ATTTACTACTGCCTTCAGCATAC | qPCR *upsX* rv |
| 2075 | GCTAGTAAAGCCAACAAGAGTG | qPCR *upsE* fw |
| 2076 | ATATAGTCGCTGCTACCCTATG | qPCR *upsE* rv |
| 2077 | TAGAGGAGCTAGCAGGAACAC | qPCR *upsF* fw |
| 2078 | ACAACATGACCGGAGTCAG | qPCR *upsF* rv |
| 2079 | TAGCCAGGGTATGTTCAGTAATC | qPCR *upsA* fw |
| 2080 | ACCTAAGTTCCCGTTATTGAC | qPCR *upsA* rv |
| 2081 | GACCAATTCGCTATCCAACTC | qPCR *upsB* fw |
| 2082 | CTGCATGTCTGATTTCCTACC | qPCR *upsB* rv |
| 3030 | GTTACGCGTAGTCCGGAACGTCATACGGGTAGGAG-CCTTCAAGGAATAATTGCTGTGCTAAG | *upsX*-HA_US_rv |
| 3031 | GAAGGCTCCTACCCGTATGACGTTCCGGACTACGCG-TAACTTTCATTTTTCTGTCTGTC | *upsX*-HA_DS_fw |
| *Saci276* | A**A**GGCCC**A**CC**G**AACCUAU | S.acidocaldarius FISH Chapter3 |
| *Sso286* | A**G**GGCCC**C**CCAA**G**CCUAU | S.solfataricus FISH Chapter 3 |
| *Stok285* | A**C**GGCCC**G**CCAAACCUAU | S.tokodaii FISH Chapter 3 |
| 1 RT_FW | ATAGGTCAAGTGATGGGTTA | |
| 1 RT_RV | CATCTGCTGCAAGTATCTTT | |
| 2 RT_FW | GCCTATACGCATGGTTTCAC | |
| 2 RT_RV | AAGGGTCAGCTAAGGGTACA | |
| 3 RT_FW | AGCAAGAAGATCACGTACTA | |

**Table S1 (all chapters).** Primers and probes used in this study. (*Continued*)

| Primer | Sequence | Purpose |
|---|---|---|
| 3 RT_RV | CTGGAGTATCCTCTATGGTAAT | |
| 4 RT_FW | GATCTAGAAGAGTTCAGTGTT | |
| 4 RT_RV | AGACCTTGGCTCTGCTTTCC | |
| 5 RT_FW | ACACAAGTGGTGAGTCAATA | |
| 5 RT_RV | TTTGCAGCGAGTTCTCCTAA | |
| 6 RT_FW | AGGGCAGTTGGCAACTTAGA | |
| 6 RT_RV | ATATCTGTGTGCTGCCGGTA | |
| 7 RT_FW | GCTGGGTGGTCTACTTTATG | |
| 7 RT_RV | AGTACTGCCCAGCAGTTA | |
| 118 FW_ NcoI | CCCCCCCATGGTACAACTAATGATGAAAGGAGG | |
| 118 RV_ BamHI | CCCCCGGATCCCGCTATTGAAGCCAGCA | |
| 117 RV_ BamHI | CCCCCGGATCCATCCGGTCCAGAGTTGA | |
| 121 FW_ NcoI | CCCCCCATGGCAATTCCAGATTTTATACTATATCAG | |
| KO-UP_ FW | CCCGGTACCGTGCGTATTATCTACGTTA | |
| KO-UP_ RV | CCCCCATGGCAGTGTTTATTTAAAGAA | |
| KO-DOWN_ FW | CCCGGATCCGGAGAATATTCATGATAC | |
| KO-DOWNS_ RV | CCCCCCCCCGCGGCCGCCGAGTGCAAAGATACTTG | |

**Chapter 2 Table S2.** Homologous *ups*-operons in other Archaea

| *S. solfataricus* | Length in aa | *S. tokodaii* | *S. acidocaldarius* | *Metalosphera sedula* |
|---|---|---|---|---|
| SSO0121 | 695 | ST1396 | Saci_1493 | Msed_2103 |
| SSO0120 | 483 | ST1397 | Saci_1494 | Msed_2104 |
| SSO0119 | 481 | ST1398 | Saci_1495 | Msed-2105 |
| SSO0118 | 154 | ST1399 | Saci_1496 | Msed_1193 |
| SSO0117 | 137 | ST1400 | Saci_1496a | Msed_2107 |

**Chapter 3 Table S2.** *S. acidocaldarius* mutants used in this study

| Strain | Genotype | Reference |
|---|---|---|
| MR31 | wild type *S. acidocaldarius* strain, Δ18 bp (position 154-172) in *pyrE* gene | Reilly & Grogan, 2001 |
| JDS28 | 335A>T in *pyrE* gene | Reilly & Grogan, 2001 |
| JDS183 | contains a frameshift mutation (duplication of T) at nt 44 of the *pyrE* gene | Reilly & Grogan, 2001 |
| SA1 | ΔSaci_*upsE* , $pyrE^-$ | This study |
| SA2 | SA1 strain with ΔSaci_*upsF* , *pyrE*$^+$ | This study |
| DG253 | derived from GA3 by 5-FOA selection, 335A>G in *pyrE* gene | This study |

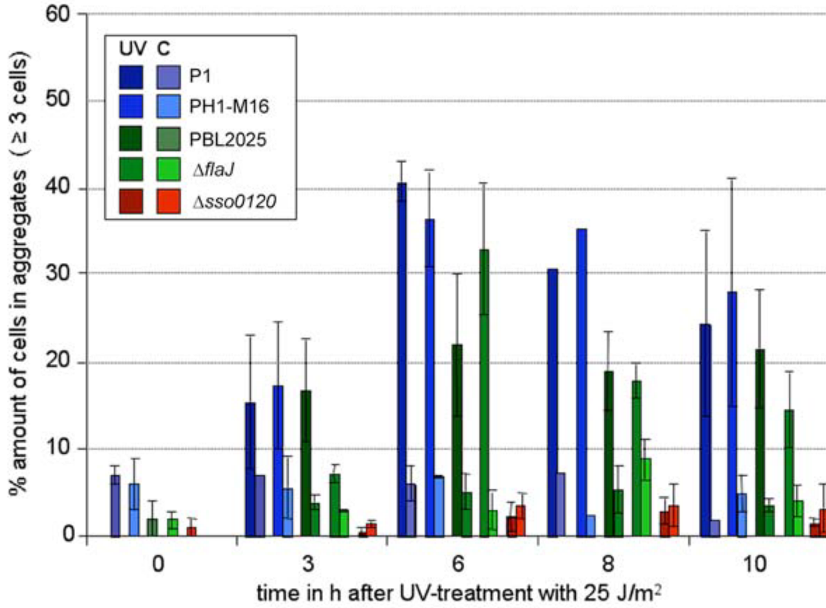
**Chapter 3 Table S3.** Recombination frequencies of S. acidocaldarius $Pil^+$/$Pil^-$ mixed matings

| ***S. acidocaldarius* mixed matings** | **Recombination frequencies** | | |
|---|---|---|---|
| | **UVxC** | **CxUV** | **CxC** |
| $Pil^+$ x $Pil^+$ | $2.87 \times 10^{-05}$ | $2.42 \times 10^{-05}$ | $3.12 \times 10^{-06}$ |
| JDS183 x JDS28 | $2.88 \times 10^{-05}$ | $1.99 \times 10^{-05}$ | $4.11 \times 10^{-06}$ |
| | $3.20 \times 10^{-05}$ | $3.32 \times 10^{-05}$ | $4.47 \times 10^{-06}$ |
| median | $\mathbf{2.88 \times 10^{-05}}$ | $\mathbf{2.42 \times 10^{-05}}$ | $\mathbf{4.11 \times 10^{-06}}$ |
| $Pil^+$ x $Pil^+$ | $1.09 \times 10^{-05}$ | $2.65 \times 10^{-05}$ | $3.93 \times 10^{-06}$ |
| JDS183 x MR31 | $2.77 \times 10^{-05}$ | $2.38 \times 10^{-05}$ | $3.05 \times 10^{-06}$ |
| | $2.57 \times 10^{-05}$ | $1.18 \times 10^{-06}$ | $2.36 \times 10^{-06}$ |
| median | $\mathbf{2.67 \times 10^{-05}}$ | $\mathbf{2.52 \times 10^{-05}}$ | $\mathbf{3.49 \times 10^{-06}}$ |
| $Pil^+$ x $Pil^-$ | $1.40 \times 10^{-05}$ | $1.46 \times 10^{-06}$ | $1.90 \times 10^{-06}$ |
| JDS183 x SA1 | $1.17 \times 10^{-05}$ | $5.95 \times 10^{-07}$ | $7.11 \times 10^{-07}$ |
| | $1.40 \times 10^{-05}$ | $1.20 \times 10^{-06}$ | $5.70 \times 10^{-07}$ |
| | $1.92 \times 10^{-05}$ | $2.13 \times 10^{-06}$ | $9.91 \times 10^{-07}$ |
| median | $\mathbf{1.40 \times 10^{-05}}$ | $\mathbf{1.33 \times 10^{-06}}$ | $\mathbf{8.51 \times 10^{-07}}$ |
| $Pil^+$ x $Pil^-$ | $1.47 \times 10^{-05}$ | $3.59 \times 10^{-06}$ | $2.59 \times 10^{-06}$ |
| JDS183 x DG253 | $2.71 \times 10^{-05}$ | $1.42 \times 10^{-06}$ | $1.79 \times 10^{-06}$ |
| | $2.21 \times 10^{-05}$ | $2.22 \times 10^{-06}$ | $3.00 \times 10^{-06}$ |
| | $2.28 \times 10^{-05}$ | $2.26 \times 10^{-06}$ | $1.95 \times 10^{-06}$ |
| median | $\mathbf{2.46 \times 10^{-05}}$ | $\mathbf{2.24 \times 10^{-06}}$ | $\mathbf{2.27 \times 10^{-06}}$ |
| $Pil^-$ x $Pil^-$ | | | |
| SA1 x DG253 | $0.00 \times 10^{00}$ | $0.00 \times 10^{00}$ | $0.00 \times 10^{00}$ |

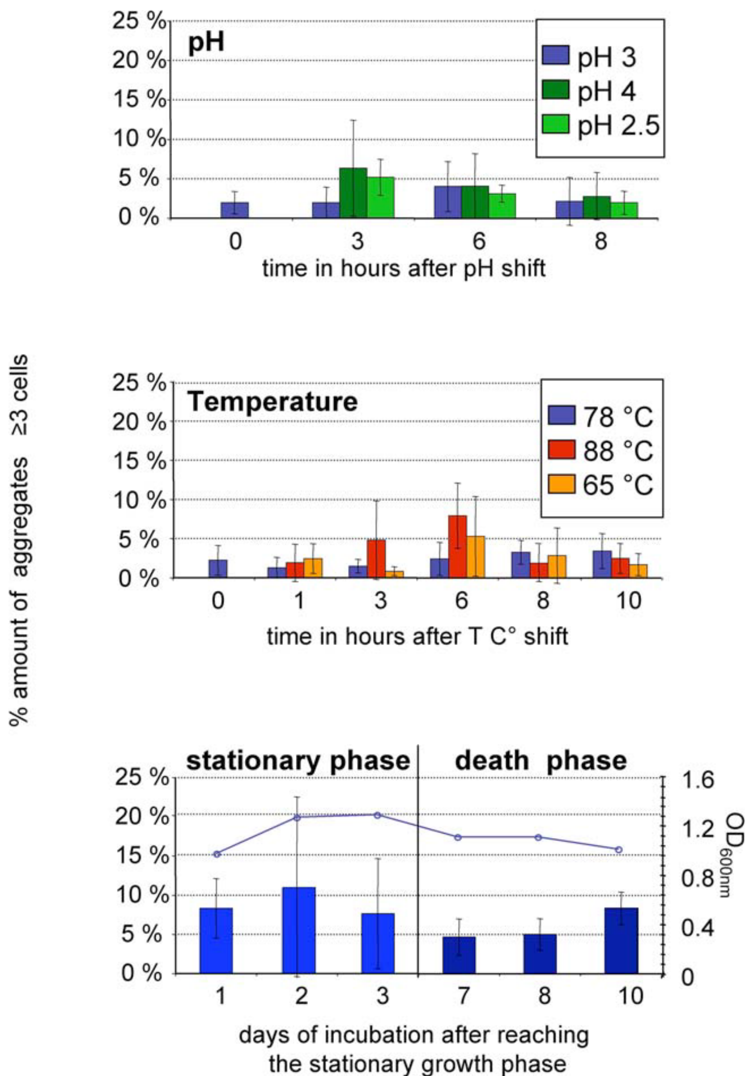
**Chapter 3 Table S4.** Post-hoc tests of differences in survival

| | **Pil$^+$ (MR31)** | **Pil$^+$ (JDS183)** | **Pil$^-$ (SA1)** | **Pil$^-$ (DG253)** |
|---|---|---|---|---|
| Pil$^+$ (MR31) | - | n.s.<br>0.586 | ***<br>< 0.001 | ***<br>< 0.001 |
| Pil$^+$ (JDS183) | n.s.<br>0.586 | - | *<br>< 0.005 | ***<br>< 0.001 |
| Pil$^-$ (SA1) | ***<br>< 0.001 | *<br>< 0.005 | - | n.s.<br>0.49 |
| Pil$^-$ (DG253) | ***<br>< 0.001 | ***<br>< 0.001 | n.s.<br>0.49 | - |

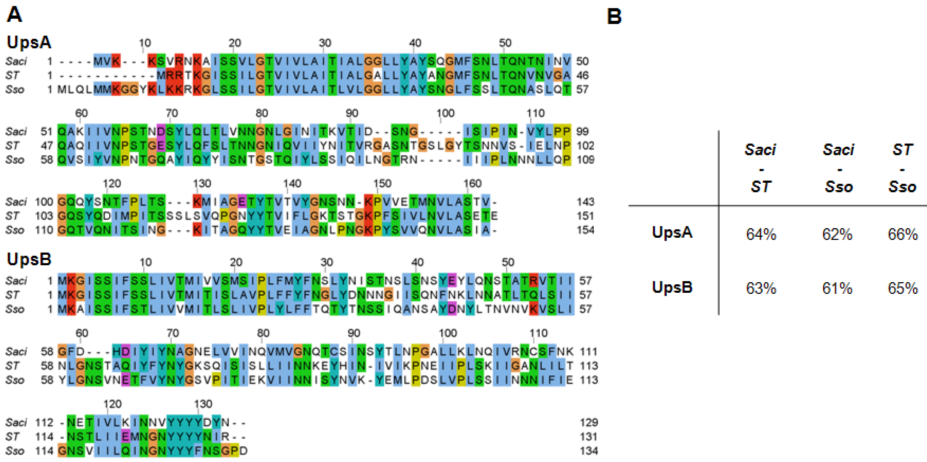
## FIGURES




**Chapter 2 Figure S1.** Quantitative analysis of the UV-induced cellular aggregation of different S. solfataricus strains. Cellular aggregation was observed at 3, 6, 8, and 10 hours after UV treatment with 25 J/m$^2$ (254 nm). The graph is based on three independent UV experiments; in the case of the strains PBL2025, Δ*flaJ* and Δsso0120 and 2 independent UV experiments for the strains P1 and PH1-M16 (only experiment at 8h, respectively). The bars display the % amount of the cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells (at minimum 500 single cells were counted, but mostly up to 1000).

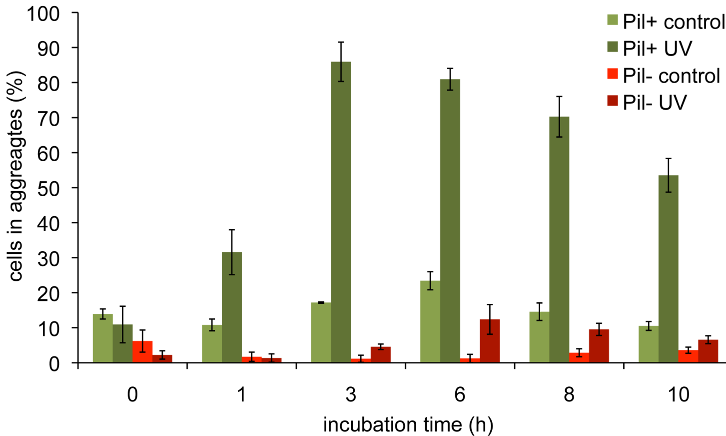



**Chapter 2 Figure S2.** Quantitative analysis of the cellular aggregation under different environmental stressors and cell growth: non-lethal pH shift, from pH 3 to pH 4 and down to pH 2.5 and a non-lethal temperature shift from 78°C up to 88°C and down to 65°C, early stationary growth phase until dead phase. The bars represent the mean of the results obtained for the four *sso0120* wild type strains; P1, PH1-M16, PBL2025 and Δ*flaJ*. The % amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells (500 single cells, or a minimum of 250 single cells were counted).

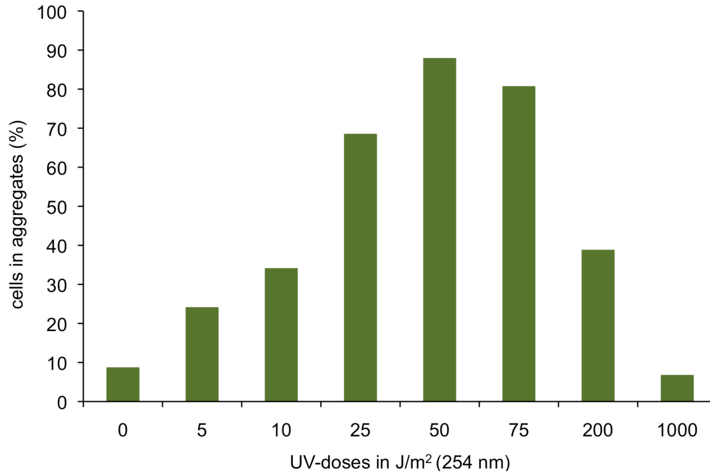



**Chapter 3 Figure S1.** Sequence alignment of the pilins UpsA and UpsB from different *Sulfolobus* species. Similar amino acids are depicted in the same color (A) with protein similarities between species (B). *Saci, S. acidocaldarius*; *ST*, *S. tokodaii*; *Sso*, *S. solfataricus.*



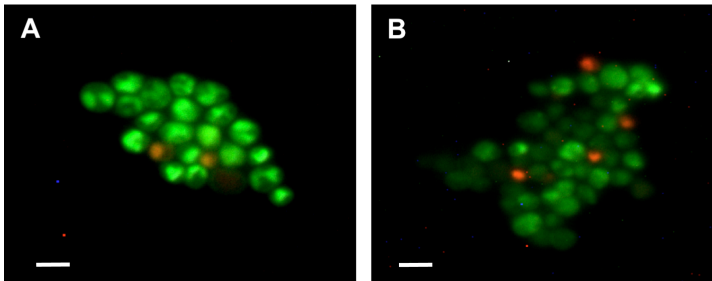



**Chapter 3 Figure S2.** Quantitative analysis of UV-induced cellular aggregation of *S. acidocaldarius* wild-type (green) and delta-pili (red) strains deficient in pili formation. The amount of cells in aggregates (>3 cells) is shown per 100 counted free cells, after irradiation with a UV-dose of 50J/m$^2$. Since most of the aggregates consisted of 10 cells or more, 30% equals an estimated minimum of 75 % of cells in aggregates, probably more. Cell clusters started to dissolve after 6 to 8 hours. Results are based on 3 independent experiments.

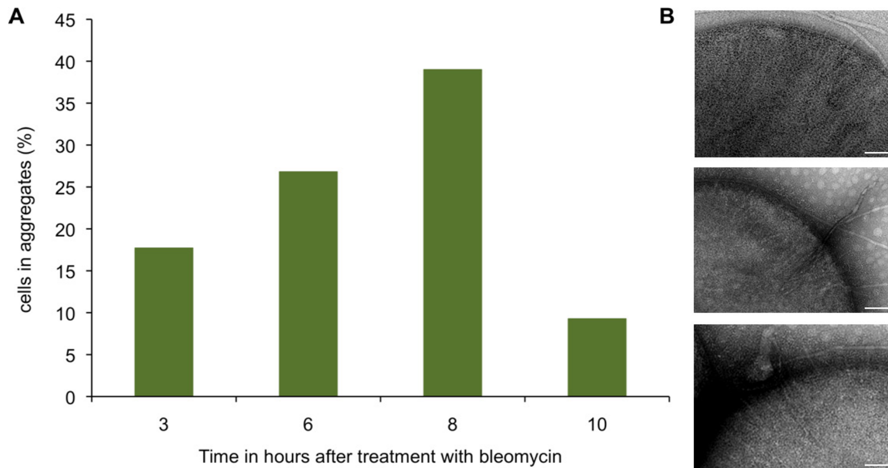



**Chapter 3 Figure S3.** Quantitative analysis of UV-dose dependent cellular aggregation in *S. acidocaldarius.* Cells were irradiated with different UV-doses and analysed after 3 hours of re-incubation at 78°C. The highest number of aggregates were found after irradiation with 25, 50 and 75 J/$m^2$.

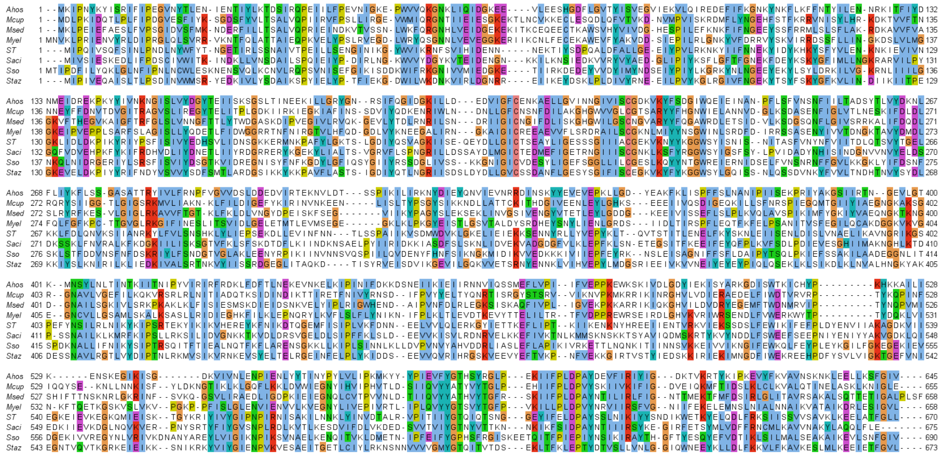





**Chapter 3 Figure S4.** Live/dead assay (Invitrogen) of cellular aggregates from *S. acidocaldarius* 3 hours after UV-treatment with 50J/$m^2$ (A) and 75J/$m^2$ (B) at 254 nm. Dead cells are in red, green cells are alive. Scale bar is 1 µm in length.




**Chapter 3 Figure S5.** Bleomycin-inducible cellular aggregation of *S. acidocaldarius* (A). Electron-microscopic analysis of pili formation 3 hours after treatment with bleomycin (B). Scale bar is 0,1 µm in length.



**Chapter 4 Figure S1.** Alignment of UpsX from different Sulfolobales; *Acidianus hospitalis (Ahos), Metallosphaera cuprina (Mcup), Metallosphaera sedula (Msed), Metallosphaera yellowstonensis (Myel), Sulfolobus tokodaii (ST), Sulfolobus acidocaldarius (Saci), Sulfolobus solfataricus (Sso), Stygiolobus azoricus (Staz).*

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## ACKNOWLEDGEMENTS

So this is the end... It's time not only to say 'Goodbye', but 'Thank You' as well. And there are a number of people who I owe to say it right now.

First of all I would like to thank my promotor Arnold Driessen. It was a pleasure to join the Molecular Microbiology group. Thank you for all scientific discussions, for help and comments, for patience for yet one more 'conjugation assay', for insightful viewing of thousands of EM pictures and for seeing ups-pili (!) over those years. Thank you also for all the advice (e.g. about oral presentations) in the most difficult moments of my PhD and for a few hours of the non-scientific talk during our trip to Marburg, I really appreciate it!

Sonja, you have been a wonderful supervisor and co-promotor. Your help, great ideas, continuous encouragement and crazy optimism were especially important in this distant supervision. It is a pity we could not continue to work in the same lab. Nevertheless, I always felt a part of your group. I was always happy to be a guest at your home and enjoyed each visit in Marburg. Thank you for being much more than a supervisor!

No man is an island... I'm so happy to meet so many friendly people during my stay in Groningen. I want to thank all former and current members of 'each year smaller' Extremophiles group. Olja and Yang, it was great time to have you next to me. Olja, thanks for many talks, support and all of the nice photos you make. Yang, you work so hard and help so many people all the time! Thank you for all the gifts, which you really shouldn't give us so often (I mean here chocolate especially!). Here I want to thank also Hyun Y. for all the 'How are you?' questions in the difficult times.

I'm grateful to all of the members of the Molecular Microbiology group, which has changed so much during my whole PhD... Thank you for all the help with my experiments, scientific and non-scientific discussions, kind words during my pregnancy (Andy -- 'I've never seen so extremely huge belly so far!'; 'Sure no twins?'). All the jokes, coffee breaks, cakes or 'after holidays' sweets left in the Secretary Corner make such a nice atmosphere in the MolMic group.

Marc and Egbert, huge part of this book has been done in the Electron Microscopy group. Thank you for giving me the opportunity to use your microscope and for all the help with specimen preparations over these years.

Marleen, we collaborated in many chapters of this thesis. Thank you for your hard work, big enthusiasm and extremely fast writing! I was also very lucky you could send me all cultures I needed :) Thank you for many friendly emails and good luck with your future work.

Special thanks for my paranimfen, two special girls Marysia and Ewa, for being my friends all this time and agreeing to be my best friends on This Day.

Jeanine, thank you for translating the summary and the scope of the thesis (!) into Dutch; Agata for correcting Polish summary and Kasia for designing the layout of the cover and all the kind emails during the final countdown.

Bea and Manon, your help with all the paperwork was invaluable.

I want to thank also all (will I be able?) the Polish people that helped me to feel here at home. Agata, Magda, Marta, Adam, Tomek, Emilia, Wiktor - it was so funny to hear mostly Polish language next to the coffee machine in the old Life Science building in Haren. Marysia, Maciek, Hania, Agata, Piotrek, Justyna, Kasia, Maciek, Marta, Robert, Magda, Asia, Cagri, Ewa, Łukasz, Jacek, Wojtek, Gabi - so many dinners, parties, trips, hours of talks - you are like a family to me! Polish Mother`s Support Group (Asia, Kasia, Marta, Łucja) - thanks for all the coffee and the Mama Dag meetings. Last but not least, Hanka, p. Isia and p. Zosia- buziak dosłownie za wszystko!

Mamo, Tato, Sławku i Jagódko, Wam dedykowana jest ta książka. Dziękuję za wiarę, co góry przenosi. Gdy ja już nie wierzyłam, Wy wciąż dodawaliście mi otuchy. Babciu, udało się! Jagusia, nigdy nie widziałam piękniejszego Sulfolobusa jak ten na okładce, wiesz?

Sławku, dziękuję za wszystkie słowa, zrozumienie, troskę, silne ramiona i morze miłości... 'Wszystko mija nawet najdłuższa żmija'*, isn`t it?



* S.J.Lec

*Gosia Ajon*

## LIST OF PUBLICATIONS

Fröls S, Ajon M, Wagner M, Teichmann D, Zolghadr B, Folea M, Boekema EJ, Driessen AJ, Albers S-V (2008) UV-inducible cellular aggregation of the hyperthermophilic archaeon *Sulfolobus solfataricus* is mediated by pili formation. Mol Microbiol 70(4):938-952

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van Wolferen M, Ajon M, Driessen AJ, Albers S-V (2013) Molecular analysis of the UV inducible pili operon from *S. acidocaldarius*. MicrobiologyOpen. Accepted

van Wolferen M, Heinrich K, Ajon M, Driessen AJ, Albers S-V. Species-specific recognition of Sulfolobales mediated by UV-inducible pili. In preparation