Chapter 5

UV-inducible cellular aggregation of the hyperthermophilic archaeon
*Sulfolobus solfataricus* is mediated by pili formation

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Abstract

The hyperthermophilic archaeon *Sulfolobus solfataricus* exhibits a complex transcriptional response to UV-irradiation involving 55 genes (Fröls et al. 2007). Among the most strongly UV-induced genes a putative pili biogenesis operon has been observed. Here we provide the first detailed description of pili in the domain Archaea encoded by an operon of six genes, including a potential secretion ATPase, two prepilins, a putative transmembrane protein and a protein of unknown function. The pili formation was inducible by UV-light (254 nm). Electron microscopy and image reconstruction showed that the pili were straight, variable in length; they were not bundled or polarised and were composed of three evenly spaced helices with 100 Å in diameter, thereby clearly being distinguishable from the archaeal flagella. Both prepilin proteins possess a class III signal sequence and the cleavage of the SSO0118 prepilin by an archaeal typeIV prepilin peptidase was demonstrated in vitro. A deletion mutant, replacing the central typeII/IV secretion ATPase (SSO0120), verified that the pili were encoded by the UV-dependently induced operon (ss00117 through ss00121) named ups-operon (UV-inducible pili operon of Sulfolobus) and that they mediated the cellular aggregation. We showed further that the cellular aggregation was a UV-dose dependent, dynamic process, not inducible by other stressors like temperature or pH, but stimulated by chemically induced double strand breaks in DNA.

We also showed that UV-irradiation strongly increases the conjugative activity of *S. solfataricus*. In the context of further transcriptional data we conclude that pili-mediated cellular aggregation probably mediates enhanced conjugation, which eventually leads to an enhanced repair of UV-damaged DNA in *S. solfataricus* via homologous recombination.

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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 et al. 1998, Battin et al. 2007, Davey et al. 2000). Cells organised in biofilm-like structures show a higher resistance to toxic compounds, as for example antimicrobials (Patel et al. 2005) or to physical stress, like shifts in temperature or pH, or exposure to UV-light (Elasri et al. 1999, Ojanen et al. 1997; Roine et al. 1998; Martinez et al. 2007). In addition, microorganisms benefit from the attachment on substrates like e.g. suspended particles, which provides a higher nutrient availability (Davey et al. 2000). Also genetic transfer, i.e. DNA exchange via conjugation plays an important role in biofilms to disseminate niche genes of metabolic pathways (Gasson et al. 1980, Molin et al. 2003). The rate of conjugative DNA exchange in biofilm structures is enhanced and conjugative pili stabilise the biofilm structure (Ghigo et al. 2001; Reisner et al. 2006; Gasson et al. 1980; Molin et al. 2003).

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. A complex biofilm-structure of a marine hydrothermal vent system was formed by methanogenic Archaea of the order Methanococcales and by Thermococcales and Archaeoglobales (Schrenk et al. 2003). Species of the order Desulfosarcina and Desulfococcus generate synergistic communities with sulphate-reducing bacteria in microcolonies (Boetius et al. 2000). An unusual microbial community organised in string-of-pearls was found in cold sulphurous water. SM1 that grows in close association with the bacterium Thiotrix sp. and forms complex and unusual cellular appendages (hami) (Moissl et al. 2003, Moissl et al. 2005). Single strain cultures of the hyperthermophilic euryarchaeote Archaeoglobus fulgidus form a protein-, metal- and polysaccharide- containing heterogeneous biofilm, which is inducible by environmental stressors like UV-light (LaPaliga et al. 1997) it is written Lapaliga in the reference list. 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).

Beside adherent multicellular structures that are found attached to diverse surfaces, non-adherent floating multicellular structures are also described. Methanosarcina mazei e.g.
forms aggregates during exponential growth (Mayerhofer et al. 1991) and halophilic archaea do so in the presence of divalent cations by (Kawakami et al. 2007, Kawakami et al. 2005). For the halophilic euryarchaeote *Halobacterium volcanii* and the hyperthermophilic crenarchaeote *Sulfolobus ssp.* cellular aggregation was observed in the context with conjugative DNA transfer (Rosenshine et al. 1989, Schleper et al. 1995, Prangishvili et al. 1998).

Characteristic for all types of cellular aggregation is the attachment between single cells, mostly mediated or stabilised by exopolysaccharides (EPS) and/or proteins (Davey et al. 2000; Kawakami et al. 2007; Klemm et al. 2004). Some microorganisms like *Xanthomonas* and *Pseudomonas*, use type IV pili to initiate or mediate the cellular aggregation (Bhattacharjee et al. 2001, Ojanen-Reuhs et al. 1996). A mutant defective in the typeIV pilus biogenesis of *Pseudomonas aeruginosa* was unable to attach on surfaces and form microcolonies (O’Toole et al. 2000, 1998). Type IV pili are also required for the twitching mobility mechanism (Mattik et al 2002), like the light regulated mobility of *Synechocystis* PCC6803 (Bhaya et al. 2001), or the coordinated cell movement in fruitbody development of *Myxococcus xanthus* (Wall et al. 1999). Type IV pili mediate in addition the DNA uptake in natural transformation systems of mesophilic and thermophilic bacteria (Averhoff et al. 2003, 2004, Graupner et al. 2001, Friedrich et al. 2003). In addition, they act as receptors for bacteriophages found in *Pseudomonas* (Roine et al. 1998).

The type IV pili biogenesis pathways and the type II protein secretion systems are very closely related, as has been demonstrated with the *E. coli* type IV pilus biogenesis secretory machinery that was able to assemble PulG, of *Klebsiella oxytoca* into pilus-like structures (Sauvonnet et al. 2000, Köhler et al. 2004).

The bacterial type IV pili machinery is also closely related to the archaeal flagella systems, as was shown by bioinformatical, biochemical and structural analyses (Peabody et al. 2003, Bardy et al. 2002, Cohne-Krausz et al. 2002, Cohen-Krausz et al. 2008, Faguy et al. 1994). The core components of the bacterial and archaeal systems are (I) a type II/IV secretion system ATPase, (II) a multispanspanning transmembrane protein and (III) the structure giving prepilins with a characteristic N-terminal signal sequence termed class III signal peptides (Peabody et al. 2003). In addition, it has been shown that the flagella of *Halobacterium salinarium* and *Sulfolobus shibatae* are in symmetry and structure more closely related to the bacterial type IV pili than to bacterial flagella. The archaeal flagellum is arranged in a three-start helical symmetry and lacks the central core (Cohen-Krausz et al.

In the genome of the crenarchaeote *Sulolobus solfataricus* three putative type IV pilus loci, containing a typeII/IV secretion ATPase, a predicted integral membrane protein and at least one ORFs containing a class III signal peptide were identified (Albers et al. 2005. The operon SSO2316, named after the central ATPase, codes for the flagellum of *S. solfataricus*. A pilus-like bundled structure of 14 nm in diameter is responsible for mobility on surfaces (Szabo et al. 2007). The operon SSO2680 encodes a recently described bindosome assembly system (Bas) that is needed for the functional surface localisation of sugar binding proteins (Zolghadr et al. 2007). The biological function of the third operon SSO0120, spanning ORFs SSO0117 through SSO0121 is unclear.

Using whole genome microarray studies to analyze the UV-response of *S. solfataricus* under UV-light of 75 J/m² at 254 nm we observed that the genes sso0117 to sso0121 were among the most highly induced genes (Fröls et al. 2007). A strong up-regulation of the operon was also observed by an independent study of M. White and co-workers using a higher UV-dose of 200 J/m², with *S. solfataricus* and *S. acidocaldarius* (Götz et al. 2008). In the same time span as the transcriptional response we observed a massive aggregation of the cells, which disappeared after the cellular regeneration (Fröls 2007).

In this study we demonstrate that extracellular pili-like structures are formed upon UV-light treatment and by using targeted deletion mutants we demonstrate that they are encoded by the UV-inducible pili operon SSO0120. Furthermore, we show that these pili-like structures are essential for the UV-dependent auto-aggregation of *S. solfataricus* cells and that this phenomenon is driven by double strand breaks in the DNA, but not by many other stress factors. In addition, UV-dependent increased conjugation events further suggest, that pili formation and aggregate formation in *Sulfolobus* mediate a repair mechanism via homologous recombination among chromosomes of sister cells.

**Material and Methods**

**Strains and growth**

*S. solfataricus* P1 (Zillig et al. 1994), M16 (Martusewitch et al.) and *S. solfataricus* PBL2025 (Schelert et al. 2004a) and the several deletion mutants were grown aerobically at 80°C in the medium described by Brock (Brock et al. 1972), adjusted to pH 3 with sulfuric acid and supplemented with 0.1(w/v) % of trypton and 0.2% (w/v) of arabinose under moderate
agitation (ca. 150rpm in NewBrunswick 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 Mg<sup>2+</sup> and Ca<sup>2+</sup> to 0.3 and 0.1 M, respectively. Plates were incubated for five days at 78°C. For the propagation of plasmids \textit{E. coli} strain DH5α was used. For the virus containing plasmids ElectroMAX™ \textit{E. coli} Stbl4™ cells (Invitrogen, Germany) were used.

\textit{Treatment with UV – light}

For the UV-treatment all preparations were performed under red dimmed light. Aliquots of 10 ml \textit{S. solfataricus} culture (OD<sub>600nm</sub> 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). The treated cultures were combined to a final volume of 20 ml. The mock treated cultures were set for 5 sec under red dimmed light. The treated cultures were stored in the dark at RT for 15 min and were re-incubated at 78°C and 150 rpm. Samples at different time points were used for microscopy, cell vitality and electron microscopy analysis.

\textit{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 x magnification at a pixel size of 5.0 Å at the specimen level with “GRACE” software (Oostergetel et al. 1996). 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 (Penczek et al. 1992; van Heel et al. 2000).

\textit{Plasmid construction for expression in S. solfataricus and E.coli}

The genes sso0117 and sso0118 were cloned 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). The expression of both genes from the resulting plasmid pSVA96 could then be induced by the addition of 0.4% arabinose to the medium of transformed cells. 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 an HA-tag to 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 co-expression 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.

**Expression of SSO0117/118 in ΔFlaJ**

For the expression of SSO117/118 ΔFlaJ was grown to an OD$_{600\text{nm}}$ of 0.2. Cells were then transformed as described by Jonscheit et al. 2003 with pSVA96. After two days the cultures were transferred to medium containing 0.4% arabinose to induce the expression of SSO0117/118. At an OD$_{600\text{nm}}$ of 0.5 cells were analyzed by electron microscopy.

**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. 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 in Albers and Driessen 2007.

**Southern blotting**

Genomic DNA (8 μg) was digested with the appropriate enzymes and separated on 0.8% agarose gel. The gel was equilibrated in 20x 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 sso120 gene were digoxigenin-labelled with the HighPrime Kit (Roche, the Netherlands). Detection was performed as recommended by the manufacturer using a LumiImager (Roche, the Netherlands).

**Expression analysis in knockout strain**

Total RNA isolation and cDNA synthesis were performed as described previously (Zolghadr et al, 2007). Gene specific primer sets (1-7) were used to detect the presence of the genes in the ups-operon. PCR products were analyzed on 0.8% agarose gels.
Growth conditions and preparation of E. coli crude membranes

BL21 (DE3) (pLysS) E. coli cells were transformed with plasmids pSW017, pSW018, pSW019 and pSW020. The cleavage assay was performed as described before in (Szabo et al, 2007a). At an OD $\text{600 nm}$ of 0.6 the expression of the precursor genes, sso0117/118, was induced by addition of 0.2% L-arabinose for 2h. Subsequently, the expression of PibD was induced with 0.1 mM IPTG (isopropyl-b-D-thiogalactopyranoside) for 2 h. The cultures were harvested the cell pellets were resuspended in 2 ml of buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA). Crude membranes were isolated as described previously (Szabo et al, 2007a) and resuspended in 50 mM Tris-HCl, pH 7.5. Cleavage of substrates was determined by SDS-PAGE and Western blot analysis of 5 μg of crude membranes. Substrate proteins were detected using monoclonal anti-hemagglutinin antibodies (Sigma).

Treatment with UV – light

For the UV-treatment all preparations were performed under red dimmed light. Aliquots of 10 ml S. solfataricus culture (OD$_{600nm}$ 0.3-0.5) were transferred to a 110 mm plastic petri dish and treated with a defined UV-dose ($\lambda$ 254 nm, UV-Stratalinker 1800, Stratagene). The treated cultures were combined to a final volume of 20 ml. The mock treated cultures were set for 5 sec under red dimmed light. The treated cultures were stored in the dark at RT for 15 min and were re-incubated at 78°C and 150 rpm. Samples at different time points were used for microscopy, cell vitality and electron microscopy analysis.

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 frequency of cells in aggregates and the amount of aggregates were counted until 1000 or 500 single cells were observed and 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 the statistic analysis the percentage of cells in aggregates $\geq 3$ 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 were analysed to observe the time and dose dependent formation of cellular aggregates in a higher resolution.
Analysis of the cell vitality

To analyse the cell vitality the LIVE DEAD Baclight™ (Invitrogen) assay was used under manufacture instructions. Alternatively, a combined DAPI propidium iodide stain was used. At 6h after UV-treatment the 20 μl liquid cultures were mixed with 2 μl propidium iodide (1:30 dilution in 10 mM Tris HCL pH 7.5) and incubated for 15 min in the dark at room temperature. Microscopic slides were coated with 1% agar (10 mM Tris HCL pH7.5) containing 0.2 μg/ml DAPI. 5 μl of the propidium iodide were spread on the coated slide and immediately examined. To analyse the amount of dead cells in aggregates in relation to the living cells in aggregates, a minimum of 50 cellular aggregates of ≥ 3 cells were counted for each UV-dose.

Testing of various stress factors

For the temperature shift, 20ml aliquots of an exponentially grown *S. solfataricus* culture (OD600nm 0.3-0.5) were transferred from 78°C to 88°C or 65°C, additionally control cultures were transferred to 78°C and 150 rpm. Samples for the quantitative analysis of the cellular aggregation were taken at 0h, 1h, 3h, 6h, 8h, and 10h after transfer.

For the pH shift, 20 ml of an exponential *S. solfataricus* culture were harvested for 10min at 4000 rpm and 4 C°. The supernatants were removed and cell pellets were resuspended in 20 ml of Brock's basal salt medium supplemented with D-arabinose (0.2%) and tryptone (0.1%) at pH 4, pH 2.5 and pH 3, respectively. Freshly inoculated cultures were incubated at 78°C and 150 rpm. Samples for the quantitative analysis of the cellular aggregation were incubated at 78°C and 150 rpm. Samples for the quantitative analysis of the cellular aggregation were taken at 0h, 3h, 6h, and 8h after pH-shift. For the treatment with the DSB (double strand break) inducing antibiotics bleomycin and mitomycin C, 20 ml of an exponential *S. solfataricus* culture were treated with 3 ug/ml bleomycin (Bleocin™, Calbiochem) or 5, 10 and 15 μg/ml mitomycin C (Sigma) and re-incubated at 78 °C and 150 rpm. The treated cultures and the control cultures were plated on Brock's solid media at 1.5h after re-incubation. Survival rates confirmed the use of a non-lethal drug concentration for both antibiotics as descript in the literature (Grogan 2001, Kosa essigmann 2004, Cannio 1998). Samples for the quantitative analysis of the cellular aggregation were taken at 0h, 3h, 3h, 6h, 8h, and 10h after drug treatment.

Conjugation assay for *S. solfataricus*

The assay was based on the transfer of the β-galactosidase encoding reporter gene lac*S* from the wildtype strain P1 to the lac*S* deficient strain M16. To distinguish both strains on plates
we selected for \textit{pyrEF} auxotrophic cells with 5-FOA. Only derivate conjugates and M16 are able to grow under these conditions and only positive conjugates would additionally form blue colonies. For each conjugation test a minimum volume of 60 ml of an exponentially grown \textit{S. solfataricus} culture (OD$_{600\text{nm}}$ 0.2–0.4) was used. The treatment was performed as described above under red dimmed light. Aliquots of 10 ml culture were poured into petri dishes and evenly spread by moderate shaking. Cultures were treated with UV light in the UV-crosslinker (254 nm, 75 or 50 J/m$^2$). Control cultures were treated under red light only. All experimental cultures had a final volume of 20 ml, for the recombination mix the \textit{S. solfataricus} strains P1 (\textit{lacS}+/\textit{pyrEF}+) and M16 (\textit{lacS}/\textit{pyrEF}–) were mixed in a ratio of 1:1. Flasks were stored in the dark for 15 min at room temperature; 40 μl of uracil (12.5 μg /ml final concentration) were added and re-incubated at 78°C for 6h and 150 rpm. Samples for plating were taken at 6h after UV-treatment and diluted in Brock's basal salt pH 3, without carbon sources.

To determine the colony forming units (cfu) without selection, cells were plated on Brock's basal salt solid media with D-arabinose (0.2%) and tryptone (0.1%) with 10 μg /ml uracil.

To analyse the reversion and mutation frequencies event/cell, the median of the cfu/ml with selection (5-FOA) were determined and divided by the median of the cfu/ml under non-selective conditions. To identify \textit{pyrEF} auxotrophic mutations of strain P1 (\textit{pyrEF}+ to \textit{pyrEF}) 200 to 800 cells, for the \textit{pyrEF} revertants of strain M16 (\textit{pyrEF} to \textit{pyrEF}+) 500 to 1500, cells were observed for each experiment. To identify \textit{lacS}+ mutations of strain P1 (\textit{lacS}+ to \textit{lacS}–) 700 to 2500 cells and revertations of strain M16 (\textit{lacS}– to \textit{lacS}+) 1000 to 3000 cells were observed by microscopy for each experiment. To determine the recombination frequency event/cell the median of the positive conjugates (\textit{lacS}/\textit{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. To identify positive ex-conjugates (\textit{lacS}/\textit{pyrEF}) cfu/ml with selection (5-FOA), in total of all UV-experiments >13000 cells were counted and 788 positive ex-conjugates were identified. In the case of the control experiments in total >11000 cells were counted and 5 conjugation events were identified, which correspond to the determined mutation frequency of P1 of $10^{-5}$ events /cell.

**Results**

**\textit{UV-inducible Induction of Operon SSO0120}**

Induction of the genes \textit{sso0118} and \textit{sso0117} belong to the strongest and fastest transcriptional reactions that were detected earlier in a genome-wide microarray study upon exposure of \textit{S.
solfataricus cells to UV-light (Froels et al. 2007). These genes belong to a cluster, and possibly an operon, of five genes (sso0117 through sso0121) all of which were very strongly induced with a maximal induction of 14-fold for sso0118 (Fig. 1). The transcriptional increases were observed at 1.5 to 5 hours after UV-treatment. A similar transcriptional reaction pattern for (sso0121, 120, 119 and 117) of these genes was observed over the time course of 8.5h hours, but not for the genes flanking upstream or downstream (sso116 and sso115, sso0122) indicating transcription from a common promoter, as suggested earlier under non-inducing growth conditions (Albers, 2005). Only gene sso0118 deviated from the UV-dependent pattern and appeared up to 3.5(6)-fold higher induced, which may indicate an additional promoter in front of the gene or alternatively, a higher stability of the transcript (Fig. 1B).

Bioinformatic analysis indicated a putative type IV pili biogenesis operon, represented by a type II/IV secretion system ATPase (SSO0120) and an integral trans-membrane protein (SSO0119) (Fig. 1A). The deduced protein sequence of the ATPase contains Walker A/B sites and the conserved domain (virB11-related ATPase COG630N) was found from position 85 to 369aa and clustered by sequence analysis into the TadA subfamilily of the type IV ATPases (Planet et al. 2001). Nine transmembrane helices can be predicted for SSO0119 and a conserved domain was found from position 109 to 456 (TadC, COG2064N). Both proteins were found to be homologous to the Tad system (TadA and TadB/TadC), which conveys non-specific tight adherence of Actionobacillus on surfaces (Kachlany et al. 2001). The last two proteins, SSO0118 and SSO0117, exhibited an N-terminal signal sequence as found in type IV pilin precursors which belong to the class III secretory signal peptides. No functional predictions could be made for the first gene, sso0121, which encodes a highly hydrophilic protein and is exclusive found in the genomes of Sulfolobales.
Figure 1. (A) Composition and characteristics of the ups-operon (UV-inducible type IV pili operon of Sulfolobus) (top) and its expression profile upon UV-treatment (bottom), extracted from whole genome microarray data (Froels et al. 2007). (B) The curves display mean values of 2 to 4 biological replicates for each time point. A UV dose of 75 J/m² at 254 nm was applied to exponentially grown cells of a S. solfataricus culture.

The putative pili operon is highly conserved in the order Sulfolobales, with the same gene arrangement in the strains S. tokodaii and S. acidocaldarius (not shown and Szabo et al., 2007a). 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 formed an operon structure whereas sso0118 was found in a different genomic region.

Maturation of prepilins
Both SSO0117 and SSO0118 contained the predicted cleavage site for the type IV prepilin peptidase PibD, an aspartyl type IV prepilin signal peptidase (Fig. 2) (Albers 2003, Szabo et al., 2007a). In SSO0117 only 6 amino acids and in SSO0118 16 amino acids would be cleaved by PibD (Fig. 2A). The ORFs of SSO0117 and SSO0118 were cloned into an E. coli expression vector already containing PibD (Szabo et al., 2007a). Using the in vivo assay the expression of the prepilin proteins was induced for two hours 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 species.
when compared to the full length protein (Fig. 2B, lane 2 and 3). Cleavage of the signal peptide of SSO0117 could not be observed, most probably because the difference between the pre-protein and the processed form does not differ enough to be separated on SDS-PAGE. Experiments to separate these two forms in IEF gel electrophoresis failed.

**Figure 2. Analysis of the pili proteins UpsA/B (SSO0117/118).** (A) The upper panel shows the N-termini of both proteins. The processing site of PibD is indicated by an arrow. (B) The lower panel shows the result of an in vivo cleavage assay of SSO0118-HA by PibD in E.coli. Expression of SSO0118-HA was detected by by Western Blot analysis using HA-tag antibodies. Lane 1: expression of SSO118-HA in the absence of PibD; lane 2: expression of SSO0118-HA two hours after arabinose induction; lane 3: 2 hrs after induction of PibD by IPTG.

**UV-induced pili formation**

We used electron microscopy to analyse cellular surfaces in order to search for pili formation upon UV treatment. To exclude that any extracellular structures were not artefacts of flagella we used the _S. solfataricus_ knock out strain ΔFlaJ that does not produce flagella (Szabo et al, 2007b). Only on the surface of the UV-treated cells, we observed pili-like structures (Fig. 3A), none were observed on untreated cells. These pili structures were spread over the whole surface and were not polarised at one cell side. Most of the cells of a UV treated culture contained many pili, some had less or very few (only 2 to 3 pili); only few cells did not expose pili on their surfaces at all. A time series experiment showed that first pili-like structures were observed at 1h after UV-treatment.

In comparison to 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 indicates that they are more fragile than flagella. Because the pili appeared straight for most of their parts, it was possible to process them by single particle analysis selecting straight segments of almost up to 1000 Å. About 700 hundred segments were extracted from long pili, aligned and summed. The final
average projection map is shown in Fig 3B. The structure appeared to be built up from three evenly spaced helices. The pitch (repeating unit) of the pili was 155 Å and the maximal diameter was about 100 Å. In the negatively stained samples the single helices appeared almost uniformly stained and there were no 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 (Froels et al. 2007). We have shown earlier that aggregation occurred with high frequency independent of the S. solfataricus genotypes, because experiments with four different strains (P1, PH1, M16, PH1 (SSV1)) showed the same phenotypic reaction. With increasing time after UV treatment, a growing number of cells were found in aggregates with the highest amount of aggregation found at 6 to 8 hours after UV treatment (Froels et al. 2007 and Fig. 7). The aggregates increased also in size, starting with three to five cells were found in the early aggregates. 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 (6h) 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 destroyed cells at all stages but not in disaggregation, indicating a high stability of the aggregates. The induction of cellular aggregation was UV-dose dependent (see Fig. 4A). We treated the cells with seven different UV doses ranging from 5 J/m² to 1000 J/m². Growth retardation of the respective cultures was directly proportional to the applied UV-dose (data not shown). Highest cellular aggregation was observed 6h after UV – treatment, i.e. at the expected maximum. The highest amount of cellular aggregation was found with 75 J/m² (at least 50 to 70% of cells were found in aggregates) whereas with a UV-dose of 50 J/m² ca. 40 to 45% of the cells were found in aggregates of ≥ 3 cells. Even the lowest dose of UV-light of 5 J/m² induced the cellular aggregation, whereas the high UV-doses of 200 J/m² and 1000 J/m² showed a very low, respectively, no significant aggregation reaction. We also observed a strong correlation between the size and amount of cellular aggregates (Fig. 3B). Low doses of 5 J/m² and 10 J/m² resulted in cellular aggregates of < 7 cells. Only upon a UV-dose of 50 J/m² to 75 J/m², big aggregates of 10 to 20 cells or more were generated frequently (average
size 12-15 cells). In the case of the high UV-doses of 200 J/m² and 1000 J/m² no aggregates larger than 4 cells were observed.

Figure 3. Electron microscopical analysis of UV inducible pili in S. solfataricus. (A) ΔFlaJ cells were analyzed by electron microscopy 3h after UV light treatment (A 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 3-stranded helical arrangement of the pili overlayed. The horizontal lines indicate the pitch of the structure, which is 155 Å.

By using different life and death staining techniques (see methods and material), we investigated if the cellular aggregation could represent an accumulation of dead cells (see Tab. 3). In the case of the lowest UV-dose of 5 J/m² 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. 64% of the total cells in the infrequent aggregates at 200 J/m² were not alive. But at lower UV-dose, like 75 J/m² even big aggregates of > 20 cells were almost uniformly constituted of living cells (Fig. 4C).

Figure 4. Aggregation of S. solfataricus cells after treatment with different UV doses (A) Quantitative analysis of cellular aggregation at 6h after UV treatment. Exponential cultures were treated with 0, 5, 10, 25, 50, 75, 200 and 1000 J/m². The % 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/m² (see asterisk), where only one experiment was performed. (B) Light micrograph of S. solfataricus cell aggregates at 6h 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 J/m² and 75 J/m². (C) Fluorescence micrograph of a S. solfataricus cell aggregate at 6h after UV treatment at 75 J/m². 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 3h after treatment. For quantitative analysis of the cell vitality at different UV doses see table 1.

Table 1

<table>
<thead>
<tr>
<th>UV dose (J/m²)</th>
<th>Amount of dead cells in % a</th>
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<tbody>
<tr>
<td>5</td>
<td>8</td>
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<tr>
<td>10</td>
<td>12</td>
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<tr>
<td>25</td>
<td>17</td>
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<tr>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>75</td>
<td>44</td>
</tr>
<tr>
<td>200</td>
<td>64</td>
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</table>

a at 6h after UV treatment a live and death stain was performed (see methods and material and also fig. 3D). A minimum of 50 aggregates ≥3 cells, were counted per each UV dose and the fraction of dead cells is given in relation to the total cells found in aggregates.

The gene products of the UV-inducible pili operon are responsible for pili formation and mediate cellular aggregation

To proof 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 the lacS gene via a double cross over. The successful knock out of the SSO0120 gene, was confirmed by Southern-analysis and RT PCR (see Fig 5A 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. After UV-treatment of the mutant ΔSSO0120 we could not observe any pili-like structures on the cellular surfaces by electron microscopy (Fig. 5C Ia and Ib). As control we used the parent strain PBL2025 (Schelert et al. 2004b), which clearly showed pili-like structures beside the flagella on its cellular surface upon exposure to UV light (Fig. 5C IIa and IIb). Overexpression of both pilin genes, sso0117 and sso0118, in ΔFlaJ strain using the virus based vector construct pSVA96 resulted in the assembly of fewer,
but extremely long and irregular pili (Fig. 6). This demonstrated that the two prepilin genes indeed form the UV inducible pili.

Figure 5. Analysis of the SSO0120 knockout strain. (A) Southern Blot analysis of wild type PBL2025 and the ∆SSO0120 strain. Genomic DNA of both strains was digested either with HindIII or EcoRI. (B) RT-PCR analysis of PBL2025 and ∆120 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 of PBL2025 (IIa and b) and ∆SSO0120 (Ia and b) 3h after UV treatment.

Figure 6. Electron micrograph of a ∆FlaJ cell overexpressing pilins, SSO117/118. (Two pictures were assembled to show the length of the pilus).

The ∆SSO0120 strain was also tested for its ability to form cellular aggregates upon UV-exposure. After a treatment with a UV-dose of 50 J/m² no significant cellular aggregation of more than 4 cells was observed (Fig. 7).

Figure 7. Quantitative analysis of UV-induced cellular aggregation of different S. solfataricus strains at 0 to 10 hours after treatment. The graph is based on four independent UV-experiments for each strain. Cellular aggregation was observed at 3h, 6h, 8h, and 10h after UV-treatment with 50 J/m² (254nm). The bars display the % amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells.
(500 to 1000 single cells were counted). No UV-induced cellular aggregation was observed in the knock out strain ΔSSO0120. Similar results were observed by using a UV dose of 25J/m² (254nm).

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 the M16 (P1 ΔlacS) showed a maximum aggregation at 6h to 8h after treatment, with an average of 45-50% cells in aggregates. In the same experiment, the PBL2025 and the ΔFlaJ strains exhibited a shifted maximum at 8h to 10h 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 Nationalpark *S. solfataricus* 98/2s (Schelert et al. 2004). Comparable results were observed when using a lower UV-dose of 25 J/m². Again, no significant cellular aggregation was observed for strain ΔSSO0120. The P1 and M16 strains showed a 30-40% lower amount of aggregation, 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 in the case of the ΔFlaJ strain the amount of cells in aggregates increased to >30% and the maximum shifted to 6h.

Based on these results we concluded that the UV-induction of the putative pilin operon, the inducible pili production and the cellular aggregation were functionally linked to each other. Based on the observed properties we named the newly identified operon **UPS** for UV-inducible typeIV pili operon of Sulfolobus, represented by the genes upsX, upsE (ATPase), upsF (TM protein), upsA and upsB (prepilins)

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

To analyse if cellular aggregation can be induced by other conditions than UV-light exposure, four strains that harbour the wild-type of the ups operon were used: *S. solfataricus* strains P1, M16, PBL2025 and ΔFlaJ. 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. PH shifts from pH 3 to pH 4 and down to pH 2.5 were similarly investigated. No significant cellular aggregation was observed under the tested conditions in
none of the four tested strains. The % amount of cells in aggregates (≥3 cells) was all below 10%. (not shown).

We also monitored the extent of cellular aggregation in the late growth phases of the cultures, from stationary to death phase (three time points for each phase). Only at the beginning of the late stationary phase, i.e. at the beginning of growth retardation, a slightly increased cellular aggregation was noted. For strain P1 up to 24% of the cells were found in aggregates of 4 to 7 cells at most, while the % amount of cells in aggregates (≥3 cells) were lower than 10% in all other growth phases (not shown).

**Cellular aggregation is induced through treatment with DSB-inducing argents**

As a response of *S. solfataricus* to UV-light we observed earlier the formation of double-strand breaks in the genomic DNA (DSB). Whereas cis-syn-cyclobutane pyrimidine dimers (CPD) represent direct DNA damages caused by UV-light effect, DSB are probably formed as a result of collapsing replication forks at unrepaired sites in the genomic DNA (Fröls et al. 2007). It has been speculated earlier, that DSB might represent an intracellular signal for further cellular reactions (Garinis et.al. 2005). We were therefore inspired to test whether the formation of DSB is connected to the formation of cellular aggregates. We tested the induction of cellular aggregation, of the different *S. solfataricus* strains P1, M16, PBL2025 and ΔSSO0120 in response to the DSB inducing argents bleomycin (Fig. 8) and mitomycin C (table 2).

**Table 2**

Cellular aggregation in % after treatment with the DNA DBS-inducing agent mitomycin C a

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sample</th>
<th>time in hours after treatment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>3h</td>
</tr>
<tr>
<td>P1 (wild type strain)</td>
<td>5 ug /ml</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>10 ug /ml</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1</td>
</tr>
<tr>
<td>PBL2025 (parent strain)</td>
<td>5 ug /ml</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15 ug /ml</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>1</td>
</tr>
<tr>
<td>ΔSSO0120 (knock out strain)</td>
<td>5 ug /ml</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>15 ug /ml</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>0</td>
</tr>
</tbody>
</table>
Chapter 5

20ml of an exponential cultures of the *S. solfataricus* strains were treated with 5, 10 and 15 μg/ml mitomycin C (Sigma) and re-incubated at 78°C and 150 rpm. The % amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells is given (500 single cells were counted).

Cells were treated with different doses of bleomycin (3 ug/ml) and mitomycin C (5, 10 and 15ug/ml) (Grogan 2001, Kosa essigmann 2004, Cannio 1998). These concentrations were non-lethal to the cells as investigated by plating efficiencies and growth behaviours in liquid cultures (data not shown). Cellular aggregation was monitored at 3h, 6h and 8h after the treatment, in the treatment with bleomycin additionally at 1h and 10h. All tested strains, except the ΔSSSO0120 strain showed a significant cellular aggregation in response to the agents. Eight hours after the treatment with bleomycin strains P1, M16 and PBL2025 exhibited 25-35 % amount of cells in aggregates (Fig. 8), while aggregation in the mock treated cultures and the bleomycin-treated strain ΔSSSO0120 remained below 10%. Similarly, although less strongly, mitomycin C induced aggregate formation in the *ups*-operon containing wildtype strains (P1, PBL2025), but not in the knock-out strain (not shown). These observations indicate that DNA damage and in particular DSBs might be a direct or indirect signal for inducing aggregate formation.

![Figure 8. Aggregate formation of different *S. solfataricus* strains after treatment with bleomycin (3 μg/ml). No significant cell aggregation was observed with the knockout strain ΔSSSO0120. The bars display the % amount of cells in aggregates (≥ 3 cells) in relation to the total amount of evaluated cells (500 single cells were counted).](image)

**UV-light induced conjugation in *S. solfataricus***

In order to study, if conjugation frequencies in *S. solfataricus* are enhanced upon UV-light exposure, we have developed a conjugation assay based on the exchange of the reporter gene β-galactosidase (*lacS*); between the *lacS* wild type strain (P1; *lacS*<sup>+</sup>*pyrEF*) and a *lacS*-deficient strain (M16; *lacS/pyrEF*). To distinguish the strains on plates, a 5-FOA negative selection was used, similar to that described by Grogan et al (1996). Only uracil auxotrophic cells were able to grow under these conditions and positive conjugants formed additionally
blue colonies when exposed to the chromophore X-Gal. To calculate the conjugation frequency, we separately determined the recombination, reversion and mutation frequencies of the mixed and single cultures, respectively (for more details see material and methods). No reversion events of the mutated pyrEF or lacS genes of the M16 (lacS/pyrEF-) strain were observed with or without UV-irradiation. The mutation frequency of strain P1 to pyrEF auxotrophy was on average $10^{-5}$/cell and experiment (similar to the determined mutation frequency for *S. solfataricus* in Martusewitsch et.al. (2000). Using a UV-dose of 75 J/m$^2$ a recombination frequency upto $1.11 \times 10^{-2}$ was observed with 1:1 mixtures of strain P1 and M16, indicating that about 1 out of 100 cells exchanged the marker upon conjugation per one generation. At 50 J/m$^2$ the conjugation reactions decreased by one order of magnitude to an observed recombination frequency of $1.09 \times 10^{-3}$. No significant conjugational activity, i.e. enhanced recombinational events, was observed without UV-treatment.

**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 typeIV 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 double stand breaks caused by UV-irradiation. We suspect that cellular aggregation mediates DNA repair *via* conjugation, as we find increase in conjugation activity upon UV-irradiation and in transcripts of genes involved in homologous recombination.

(i) 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 1h after UV-treatment and reached a maximum within 5 to 6 hours. To proof 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 et al. 2007) to produce a specific knockout of the putative secretion ATPase SSO0120. No pili structures were observed on the cellular surface of the ΔSSO0120 strain.

By testing the ΔSSO0120 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 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. 2007).

These pili like structures are spread over the whole cellsurface. They are not bundled or polarized like the cable-like flagellar-bundles of *Pyrococcus 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 (Kachalany 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 death stain analysis (fig 4A).

The detailed mechanism 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 et al. 2002). 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.

(ii) UV-inducible cellular aggregation is highly dynamic.

A quantitative assay was developed in this study to analyze 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 3-5 cells accumulate, which later aggregate to bigger forms. The maximum of aggregation was reached at 6h to 8h after UV treatment, followed by a clear disappearance, interpreted as an active dissociation. 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. Maxi-aggregates with even up to 100 and more cells were found frequently at 6h 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 \textit{S. solfataricus}, which is around 7h, the dynamics of this process is relatively fast. For example, the cellular packets of \textit{M. mazei} need 2 to 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. 1991). The stress induced biofilm formation of \textit{Archaeoglobus fulgidus} occurs in 2-12 hours. But in this case no disaggregation was reported (LaPaglia et al. 1997).

(iii) 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. 1996, Roine et al. 1998, Martinez et al. 2006). Treatment of the hyperthermophilic archaeon \textit{Archaeoglobus fulgidus}, with a high dose of UV-light and other physical or toxic stressors results in a biofilm production. (LaPaliga et al. 1997). Mutants defect in the auto-aggregation of the plant pathogen bacteria \textit{Pseudomonas syringae} and \textit{Xantomonas campestris} showed a higher sensibility to UV-irradiation, than the wildtype capable of forming multicellular structures (Roine et al. 1998, Ojanen-Reuhs et al. 1996). A decreased sensitivity to UV-light and other environmental stressors was also reported for biofilms of the yeast-like fungi \textit{Cryptococcus neoformans} (Martinez et al. 2007). None of the stressors that we used for \textit{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 \textit{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 % amount cells involved) was dependent on the UV-dose. Relatively high doses of UV-light, like 200 J/m² or 1000 J/m² resulted in an unsignificant amount of small aggregates (≤4 cells) and killed most of the cells. In contrast a relatively low dose of UV-light, like 5 J/m² induced cellular aggregations. In Nature sunlight with ca. 96\% UVA and 4\% UVB reaches the ground and is the most DNA damaging factor. The daily dose of DNA-damaging UVB light on a sunny day in the northern and southern world hemispheres is measured between 1-3 kJ/m² over 24h (depending on the season). The experimentally used UVC (254 nm) is about 100 fold more effective than UVB in inducing CPDs (Kuluncsics et al. 1999). With reference to the observation that even low dose of UV-light significantly induce the cellular aggregation of \textit{S. solfataricus} we conclude
that this phenotypic effect reflects the behaviour of the organism to the sunlight in the natural environment.

(iv) Cellular aggregation is induced by double strand breaks and might mediate a recombinational repair system via conjugation.

Between 2 and 8 hours after UV treatment we observed the formation of DNA double-strand breaks (DSB), probably resulting from replication fork collapse at CPD 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 DSBs in the genome. Indeed, the DSB inducing argents Bleomycin and Mitomycin C caused the same phenotype of cellular aggregation as UV-light. Similarly, the proliferation of the *Sulfolobus 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 *Halobacterium 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, both such components are not 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).

During our experiment with the DSB inducing argents we observed that the deltaSSO0120 strain reacted more sensitively to the treatment than the *ups*-wildtype strains. This leads to the hypothesis that the cellular aggregation is needed for an efficient DNA-repair. But it could as well be possible that aggregation is acting as efficiently as a physical protection mechanism simply providing shade within the tight cellular consortium.

However, several lines of evidence indicate, that cellular aggregation might play an important role for mediating conjugation-dependent DNA repair via homologous recombinations:

(I) Cellular aggregation of *Sulfolobus* has previously been reported in the context of conjugation mediated by plasmids (Schleper et al. 1995). The structure of the cellular aggregates was highly similar to the here described UV-caused phenotype.

(II) We have observed UV-light enhanced conjugative activity in this study at the same UV-dose ranges as used for inducing cellular aggregation and with a frequency that correlated with the applied UV dose.

(III) UV-inducible conjugation has been described earlier for *S. acidocaldarius*. After UV-
treatment with 70 J/m² the highest recombination frequencies were reached (between $10^{-4}$ to $10^{-1}$) (Wood, 1997).

(IV) We found a slight, but significant up-regulation 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 (Constantinesco et al. 2004, Hopfner et al. 2002).

By integrating our observations 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 in more detail the nature of the cell-cell-contacts, e.g. if exo-polysaccharides are produced to stabilize the complexes and if aggregation can occur among closely related species. 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.

Although the specific UV-inducible operon involved in pili formation in *Sulfolobus* is not conserved outside the order Sulfolobales, we consider it likely that cellular aggregation and enhanced conjugation might be a more general principle employed by microorganisms that are regularly exposed to sunlight.

References


Chapter 5


