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The *mef*(A)/*msr*(D)-carrying streptococcal prophage Φ1207.3 encodes an SOS-like system, induced by UV-C light, responsible for increased survival and increased mutation rate

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Abstract

Bacterial SOS response is an inducible system of DNA repair and mutagenesis. Streptococci lack a canonical SOS response, but an SOS-like response was reported in some species. The *mef*(A)-*msr*(D)-carrying prophage Φ1207.3 of *Streptococcus pyogenes* contains a region, spanning *orf6* to *orf11*, showing homology to characterized streptococcal SOS-like cassettes. Genome-wide homology search showed the presence of the whole Φ1207.3 SOS-like cassette in three *S. pyogenes* prophages, while parts of it were found in other bacterial species. To investigate whether this cassette confers an SOS-mutagenesis phenotype, we constructed *Streptococcus pneumoniae* R6 isogenic derivative strains: (i) FR172, streptomycin resistant, (ii) FR173, carrying Φ1207.3, and (iii) FR174, carrying a recombinant Φ1207.3, where the SOS-like cassette was deleted. These strains were used in survival and mutation rate assays using a UV-C LED instrument for which we designed and 3D-printed a customized equipment, constituted of an instrument support and swappable-autoclavable mini-plates and lids. Upon exposure to UV fluences ranging from 0 to 6400 J/m^2 at four different wavelengths, 255, 265, 275 and 285 nm, we found that the presence of Φ1207.3 SOS-like cassette increases bacterial survival up to 34-fold. Mutation rate was determined by measuring rifampicin resistance acquisition upon exposure to UV fluence of 50 J/m^2 at the four wavelengths by fluctuation test. The presence of Φ1207.3 SOS-like cassette resulted in a significant increase in the mutation rate (up to 18-fold) at every wavelength. In conclusion, we demonstrated that Φ1207.3 carries a functional SOS-like cassette responsible for an increased survival and increased mutation rate in *S. pneumoniae*.

Importance

Bacterial mutation rate is generally low, but stress conditions and DNA damage can induce stress response systems, which allow for improved survival and continuous replication at the cost of elevated mutagenesis. The SOS response is a DNA repair mechanism activated by some bacteria in response to stressful conditions, which leads to a temporary hypermutable phenotype and is usually absent in streptococcal genomes. Here, using a reproducible and controlled UV irradiation system,

we demonstrated that the SOS-like gene cassette of prophage Φ1207.3 is functional, responsible for a temporary hypermutable phenotype, and enhances bacterial survival to UV irradiation. Prophage Φ1207.3 also carries erythromycin resistance genes and can lysogenize different pathogenic bacteria, constituting an example of a mobile genetic element which can confer multiple phenotypes to its host.

Introduction

Bacteria evolve through chromosomal rearrangements, acquisition of DNA by horizontal gene transfer and mutation. Mutations arise by a process referred to as mutagenesis which is measured determining the mutation frequency or the mutation rate. Mutation frequency represents the proportion of mutant cells in a specific culture, while the mutation rate is the probability of a mutation occurring per cell division and thus is also an estimation of the probability of a mutation occurring during the life of a bacterial cell (1–3). Mutation rates differ among bacterial species and among strains of the same species, and are influenced by the fidelity of DNA replication, DNA damage and repair pathways, and exposure to chemical or physical agents. In *Escherichia coli* DNA replication is carried out by high fidelity polymerases which misinsert nucleotides at a rate of 10^{-4}-10^{-6} per base pair (bp), but possess a 3'-exo proofreading activity that reduces mutation load by about 100-fold (4). *E. coli* has also evolved the SOS response, which is inducible by DNA damage and allows for improved survival and continuous replication at the cost of elevated mutagenesis (5, 6). The main proteins involved in SOS response are LexA, a homodimeric transcriptional repressor of the SOS regulon, and RecA, which, upon DNA damage, binds to ssDNA, becomes activated, and promotes the autocleavage of LexA and subsequent transcription of the SOS genes (7). The SOS response induces the expression of genes involved in DNA-damage tolerance and repair, including the Translesion Synthesis (TLS) system (8). TLS is involved in the tolerance to DNA damage, and can be both error-free or error-prone, when the polymerase V is recruited. Polymerase V is encoded by the *umuDC* operon, where *umuC* encodes a translesion DNA polymerase, of the Y family, while

umuD encodes a regulatory protein. The low fidelity polymerase V has a high rate of nucleotide misincorporation (10^{-3}-10^{-4} per bp) (9) and also lacks proofreading activity (10).

Streptococci lack a canonical SOS response (11), but SOS-like gene cassettes are present in the genomes, either on the chromosome or on Mobile Genetic Elements, of *Streptococcus uberis, Streptococcus agalactiae, Streptococcus pyogenes, Streptococcus sanguinis, Streptococcus mitis,* and *Streptococcus thermophilus* (12). In *S. uberis*, the SOS-like cassette contains 4 genes arranged in an operon including (i) *hdiR*, coding for a protein homologous to the HdiR regulator of *Lactococcus lactis*, induced by heat shock and DNA-damage (13), (ii) *umuC* encoding an UmuC-like protein, (iii) 2 genes of unknown function. The HdiR regulator of *S. uberis* undergoes spontaneous self-cleavage at alkaline pH, similar to the RecA-mediated self-cleavage of LexA-like proteins. HdiR specifically binds to an inverted repeat sequence downstream the *hdiR* putative promoter, suggesting a role as transcriptional repressor of the SOS-like cassette. The pneumococcal Integrative and Conjugative Element (ICE) Tn*5253* confers UV resistance to the cell, inducing a mutagenic SOS response (14). Tn*5253* contains an SOS-like cassette similar to that described in *S. uberis*, comprising: (i) *orf69*, which codes for an LexA-like repressor, (ii) *orf70*, which codes for an UmuC-like protein, (iii) *orf71* and *orf72* of unknown function (15–17). In *S. pyogenes,* a gene cassette homologous to the already known streptococcal SOS-like cassettes is associated with the *mef*(A)-*msr*(D)-carrying prophages Φ1207.3 (18–21), Φ10394.4, Φm46.1 and its variant VP_00501.1 (22–25). Φ1207.3 is a functional bacteriophage capable to lysogenize different strains of the human pathogens *S. pyogenes* and *S. pneumoniae*, which colonize the upper respiratory tract (26). Since both of these species can cause invasive infections, the transmission of antibiotic resistance genes by lysogenic transfer of Φ1207.3 assumes clinical relevance. In this work, we transferred Φ1207.3 prophage from the original *S. pyogenes* host to the *S. pneumoniae* standard laboratory strain R6 to investigate if the SOS-like cassette carried by the Φ1207.3 prophage is able to activate an SOS-like response, which could increase the acquisition of mutations associated to antimicrobial resistance.

Materials and methods

Bioinformatic analysis

Homology searches were conducted in public nucleotide and protein databases, namely Microbial Genomes Database (https://www.ncbi.nlm.nih.gov/genome/microbes/), Conserved Domain Database (CDD, https://www.ncbi.nlm.nih.gov/cdd/) and Pfam protein family database (https://pfam.xfam.org/), using default parameters, and only alignment with E-values <0.001 were considered. Protein sequence analysis was conducted with the tools available at Softberry website (http://www.softberry.com/berry.phtml) and with the software Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (27). Nucleotide sequence of the orf6-orf11 region was used as a probe for Microbial Genomes Database searches, while ORF predicted amino acid sequences were used for protein databases searches.

Bacterial strains and growth

Streptococcal strains used in this work and their relevant properties are reported in Table 1. Strains were grown in Tryptic Soy Broth (TSB, BD) at 37°C. Starter cultures were taken at an optical density at 590 nm (OD_{590}) ranging from 0.2 to 0.3 and were frozen in 10% glycerol at -70°C. Solid media were obtained by supplementing TSB with 1.5% agar (BD Difco) and 3% defibrinated horse blood (Liofilchem). When required, both liquid and solid media were supplemented with antibiotics at the following concentrations: 500 µg/ml kanamycin, 500 µg/ml streptomycin, 0.5 µg/ml erythromycin, 5 µg/ml chloramphenicol and 20 µg/ml rifampicin.

Lysogenization assays

Transfer of bacteriophage Φ1207.3 was obtained through a plate mating experiment, as previously described (28). Briefly, donor cells, carrying the phage, and recipient cells were grown separately in TSB in the presence of the appropriate antibiotics. Upon reaching the end of the exponential phase, cells were mixed at a donor-recipient 1:10 ratio, centrifuged, and the pellet was plated on TSA plates supplemented with 5% horse blood. Plates were incubated at 37°C in the presence of 5% CO_2 for 4h, cells were then recovered with a cotton swab and resuspended in TSB/10% glycerol. To

select for lysogens, cell suspension was plated following a multilayer plating procedure in presence of erythromycin and streptomycin which select for lysogens and counterselect the Φ1207.3 donor cells. To confirm the lysogens phenotype, a genetic analysis was performed as reported (16,28), while PCR and direct Sanger sequencing of PCR template were used for genotype analysis according to the protocol already described (32).

PCR Gene SOEing mutagenesis

To generate a knock-out mutant of *orf6-orf11* region, PCR Gene Splicing by Overlap Extension (SOEing) was used to produce a mutagenic construct containing the kanamycin resistance cassette (876 bp) flanked by the DNA fragments located upstream of *orf6* (555 bp) and downstream of *orf11* (829 bp) as previously described (29). The primer pair IF149/IF210 was used to amplify the 876-bp *ami/aphIII* kanamycin resistance cassette (30), while IF242/IF239 and IF240/IF241 amplified the flanking fragments. Assembly of the final construct (2,260 bp) was obtained with the IF242/IF241 primer pair. PCR was carried out as previously described (31). The PCR products were separated in a 0.8% agarose gel at 200 V for 30 minutes, stained for 15 minutes in a 0.1% Ethidium Bromide solution and visualized with UV light. PCR products after gel purification (NucleoSpin Gel and PCR Clean-up kit, Macherey-Nagel) were quantified using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Oligonucleotide primers are listed in Table S1. The PCR mutagenic construct was directly used as donor DNA in transformation experiments. , Since integration of Φ1207.3 into *S. pneumoniae* chromosome leads the disruption of the *celB* competence gene with a consequent impairment of genetic transformation, we constructed transformable *S. pneumoniae* Rx1 derivative strains carrying Φ1207.3 integrated elsewhere in pneumococcal chromosome to be used as tranformation recipients (Table 1) (28). Pneumococcal competent cells were prepared as previously described (29). Competent cells were thawed, supplemented with 25 ng/ml Competence Stimulating Peptide (CSP) and 1 μg/ml of the purified mutagenic construct, and the transformation mixture was incubated at 37°C for 45 minutes. The resulting recombinant strains were selected for acquisition of kanamycin resistance by multilayer plating (28). The deletion of the *orf6-11* region (nucleotides

6,189 to 9,297, GenBank accession no. AY657002) was confirmed by PCR and direct sequencing of the PCR products (32).

UV fluence measurement

UV-C light experiments were carried out using a PearlLab Micro UV-C LED instrument (AquiSense Technologies, Erlanger, Kentucky, USA). The instrument is a compact device equipped with 4 interchangeable USB UV-C LEDs emitting at different wavelengths (255, 265, 275 and 285 nm) and a dimmer switch that allows irradiation with 10 different intensities. For our purposes, the lower portion of the instrument was un-screwed, and the lamp was used as a general light source. A customized equipment, constituted of an instrument support and swappable and autoclavable mini-plates and lids, was 3D-designed and printed in Nylon-12 (Figure S1, https://doi.org/10.5281/zenodo.8208699). The irradiance was measured 3 times with a ILT1400 radiometer and SED240/W detector (International Light Technologies, Peabody, MA). Mean and standard deviation for each intensity of the different wavelengths used are reported in Table S2. The UV fluence (J/m^2) is calculated as "irradiance (W/m^2) x exposure time (sec)", thus the same value of the UV fluence can be obtained varying the values of the 2 parameters. Since the PearlLab Micro UV-C LED instrument emits light at 10 different intensities, allowing for 10 different values of irradiance for each of the 4 wavelengths, different combinations of intensity and time were possible. The choice of the optimal parameters to be used in UV-C light survival and fluctuation assays was based on a preliminary survival experiment where an UV fluence of 50 J/m^2 was obtained varying the irradiance and the time. Since the combination of high intensities and low exposure times, or low intensities and high exposure times, resulted in a wide variability of survival among the technical replicates, we chose, when possible, to maintain similar exposure times across all wavelengths and to use intermediate intensities for the 50 J/m^2 UV fluence for fluctuation assays (Table S3).

Survival assay

Bacterial survival upon UV-C light irradiation at different wavelengths (255 to 285 nm) was assessed by plating the cells after exposure to different UV fluences from 0 to 6400 J/m^2 (0, 25, 50, 100, 200, 400, 800, 1600, 3200, 6400) and by calculating the cell viability for each strain. Cells were grown at 37°C in 10 ml of TSB until reaching an OD_{590} of 0.6 (about 1 x 10^8 CFU/ml), when they were centrifuged at 5,000 x g for 15 minutes, washed with sterile phosphate buffered saline (PBS, Sigma Aldrich) and resuspended in 10 ml of PBS. UV-C survival experiments were carried out by placing 1 ml of cells in the sterile Nylon-12 mini-plate and exposing them to different fluences, under continuous stirring. UV-C irradiated and untreated control bacterial cells were plated by multilayer plating. Survival was calculated as the ratio between UV-C irradiated and not irradiated control cells.

Luria-Delbrück fluctuation test

Mutation rate was determined by fluctuation analysis in absence or presence of UV-C light at different wavelengths ranging from 255 to 285 nm. Strains were grown in TSB at 37°C until an OD_{590} of 0.6 and the number of total and rifampicin-resistant CFUs was determined prior to freezing at -70°C in 1 ml-aliquots containing 10% glycerol. Rifampicin was used at a final concentration of 20 µg/ml in the third layer of TSA, when appropriate (28). For mutation rate determination, a set of 10 independent cultures was obtained by inoculating 5 ml of TSB with 1 x 10^5 CFU of the same 1 ml frozen aliquot. For mutation rate determination following UV-C light treatment, the 1 ml frozen aliquot was irradiated at an UV fluence of 50 J/m^2 before inoculum. Then, to prepare 10 independent cultures, an inoculum corresponding to 1 x 10^5 CFU was calculated by inferring bacterial viability after irradiation from the data obtained with the UV-C light survival assay. Cultures were grown at 37°C until they reached the mid-log phase, corresponding to about 12 generations, the number of total and rifampicin-resistant CFUs was determined by plating 0.1 ml of culture and incubating the plates at 37°C for 48 h. For each strain and condition, 4 replicates of the fluctuation test were carried out and the rifampicin-resistant CFUs numbers of the 40 cultures were pooled into a single data set (33). The mutation rate determination and the statistical analysis were

carried out using the R package rSalvador (34). The expected number of mutations per culture (m) was first obtained with the use of the function *newton.LD.plating*, which computes the maximum likelihood estimates (MLE) of m, adjusting to the plating efficiency, i.e., when only a portion of the culture is plated. Once the estimate of m was obtained, the mutation rate (μ) was determined dividing m by the number of cell divisions that had taken place. Since the final number of cells in a culture, N_t, arises from N_t-1 divisions, the mutation rate is:

$$\mu = \frac{m}{Nt - 1} \approx \frac{m}{Nt}$$

where N_t -1 is equal to the number of generations and can be approximated to N_t (3, 34). Differences in mutation rates were compared using the rSalvador likelihood ratio test (LRT) (34–36), which takes into consideration both the plating efficiency and the final cell number N_t, which differs among different strains or experimental conditions, by applying the *compare.LD.plating* function pairwise among all conditions of each strain and among the different strains. The *p.adjust* R function was then used to correct the overall false positive rate of the obtained p values for multiple comparisons. Differences in the mutation rate were considered significant only when the adjusted *p*-values were less than 0.05 (33). The comparison was also observed graphically by checking the overlapping of the 84% confidence intervals (C.I.s) (36).

Results

Φ1207.3 carries an SOS-like cassette

The Φ1207.3 DNA region located between the *mef*(A)-*msr*(D) erythromycin resistance cassette (20) and the type II restriction-modification cassette (37), contains 6 open reading frames (namely *orf6* to *orf11*) likely arranged in an operon (Figure 1). Homology search revealed that *orf11* predicted gene product is homologous to the HdiR LexA-like transcriptional repressor of *Lactococcus lactis*, *orf8* is homologous to the *Streptococcus pneumoniae* Tn*5253 umuC/orf70,* while *orf7* gene product is homologous to the *Bacillus subtilis* YolD-like protein, which is predicted to be a functional equivalent of UmuD (38) (Table 2). The deduced amino acid sequences of *orf6* to *orf11* share

homology (40-73%) with the predicted gene products of the *S. uberis* and of the *S. pneumoniae* Tn*5253* SOS-like cassettes, respectively (12, 14), with the exception of *orf9* and *orf10*, which appear to be unique for Φ1207.3. For these reasons, the *orf6* to *orf11* region of Φ1207.3 was referred to as an SOS-like cassette.

Presence of the Φ1207.3 SOS-like cassette in bacterial genomes

A genome-wide homology search of the Φ1207.3 SOS-like cassette in the 67,343 complete bacterial genomes available in the GenBank Microbial Genomes Database (accessed in March 2022) revealed the presence of the cassette, or parts of it, in 104 complete bacterial genomes (Figure S2, Table S4). In 3 *S. pyogenes* genomes, a cassette 100% homologous to that of Φ1207.3 was found associated to *mef*(A)-*msr*(D)-carrying prophages, including Φ10394.4. A copy of the SOS-like cassette, containing a short deletion at the 5' end of *orf11*,was found in 12 additional streptococcal genomes, including *Streptococcus gallolyticus*, *Streptococcus dysgalactiae*, and *Streptococcus suis*. In the remaining 89 genomes, fragments of the cassette were found, including a fragment spanning *orf6* to *orf9* in 6 *S. suis* genomes and a fragment spanning *orf6* to the partial 3' end of *orf8* in 43 genomes, including 16 pneumococcal genomes. The fragment belongs to the *mef*(A)-*msr*(D)-carrying element Tn*1207.1* (39, 40) in the *S. pneumoniae* INV200 genome and to the *mef*(E)-*msr*(D)-carrying mega element (41, 42) in the remaining 15 genomes.

Construction of isogenic S. pneumoniae recombinant strains

To investigate if the presence of this cassette confers an SOS-mutagenesis phenotype we constructed three isogenic derivative strains of the *S. pneumoniae* standard laboratory strain R6: (i) FR172, streptomycin resistant derivative of R6, (ii) FR173, derivative of FR172 carrying Φ1207.3, and (iii) FR174, derivative of FR172 carrying a recombinant Φ1207.3, where a 3109-bp DNA fragment, corresponding to the *orf6* to *orf11* region, was deleted by substitution with a kanamycin resistance cassette (Table 1). Isogenic strains were constructed in R6 since it harbours a functional Mismatch Repair System (*hex*+) which results in a low basal mutation rate (43–46).

The presence of the Φ1207.3 SOS-like cassette increases bacterial survival upon UV-C light irradiation

To assess whether the SOS-like cassette of Φ1207.3 is involved in increased survival upon UV-C light treatment, the bacterial viability of the three isogenic strains was measured upon exposure to UV fluences ranging from 0 to 6400 J/m^2 at four different wavelengths, 255, 265, 275 and 285 nm (Figure 2). No significant difference in bacterial survival was observed between strain FR172, not carrying Φ1207.3, and FR174, carrying the recombinant Φ1207.3Δ*orf6-orf11* devoid of the SOS-like cassette. The presence of the Φ1207.3 in FR173 increases bacterial survival upon UV-C irradiation compared to FR172 and FR174, regardless of UV-C light fluences and wavelengths. Noticeably, at a 6400 J/m^2 fluence we did not observe any increase in survival due to the SOS-like cassette presence. It is likely that, at high UV fluences, the accumulation of DNA damages with the associated high rate of mortality hinders the activation of the SOS-like response. FR173, carrying Φ1207.3, resulted in an increased bacterial survival compared to FR172, not carrying Φ1207.3, up to (i) 8.46-fold at 255 nm, (ii) 29.11-fold at 265 nm, (iii) 16.10-fold at 275 nm, (iv) 7.26-fold at 285 nm. When compared to FR174, carrying the recombinant Φ1207.3ΔSOS-like cassette, FR173 resulted in an increased bacterial survival up to (i) 10.93-fold at 255 nm, (ii) 34.21-fold at 265 nm, (iii) 10.69-fold at 275 nm, (iv) 8.23-fold at 285 nm (Table S5).

The Φ1207.3 SOS-like cassette increases mutation rate upon UV-C light irradiation

Activation of the Φ1207.3 SOS-like response upon UV-C exposure was assessed determining the mutation rate by measuring the acquisition of rifampicin resistance, associated to point mutations in the *rpoB* gene coding for the β subunit of RNA polymerase (47, 48). A significant increase of mutation rate was observed at every wavelength tested in FR173, carrying Φ1207.3, whereas no significant increase was observed in the FR172, not carrying Φ1207.3, and FR174, carrying the recombinant Φ1207.3ΔSOS-like cassette (Figure 3). In particular, in FR173 the mutation rate increase of irradiated cells compared to not irradiated cells was: (i) 6.64-fold at 255 nm (p-value = 0), (ii) 9.50-fold at 265 nm (p-value = 0), (iii) 8.63-fold at 275 nm (p-value = 0), and (iv) 18.38-fold

at 285 nm (p-value = 0), whereas in FR172 and FR174 no significant increase in mutation rate was observed (Table S6). In absence of UV-C light irradiation, the three isogenic strains showed similar mutation rate values, regardless of the presence of the Φ1207.3 prophage or of the SOS-like cassette.

Discussion

Mutation rate is generally low in bacteria, but hypermutable phenotype can arise as a consequence of the loss of DNA proofreading during replication Sundin and Weigand 2007; Gressel and Levy 2010; Jolivet-Gougeon et al. 2011). Stress conditions and DNA damage can also induce stress response systems, which allow for improved survival and continuous replication at the cost of elevated mutagenesis (6, 53). The SOS response is a DNA repair mechanism activated by some bacteria in response to stressful conditions, which leads to a temporary hypermutable phenotype (5, 6). In the present work, we demonstrated that the *S. pyogenes* prophage Φ1207.3 carries a functional gene cassette responsible for the activation of an SOS-like response in *S. pneumoniae* in response to UV-C light irradiation. We studied this cassette after its introduction in the *S. pneumoniae* R6 strain, which contains a Mismatch Repair System (*hex*) conferring a low mutation rate (43). We observed that the activation of this SOS-like cassette, upon UV-C exposure, makes the R6 strain temporarily hypermutable, resulting in a higher ability of surviving and evolving. Hypermutable strains have been found in pathogenic bacteria (49) and represent a huge problem in clinical settings (54–57). Clinical strains are frequently exposed to variable and changing stressful conditions, such as antibiotic treatments and host defenses, which can lead to evolution of resistance and virulence phenotypes, with consequent therapeutic failures (33, 51, 58–62). Hypermutable strains were also characterized in *S. pyogenes*, where the DNA Mismatch Repair (MMR) gene *mutL* can be interrupted by the insertion of a genomic island. This genomic island is capable of growth-phase-dependent excision and functions as a switch for the hypermutator phenotype (63). Prophage Φ1207.3, originally found in a clinical isolate of *S. pyogenes*, carries the erythromycin resistance genes *mef*(A)-*msr*(D) (18–21) and is able to transfer among *S. pyogenes* strains with different

genetic background (unpublished data) and other streptococcal species such as *S. gordonii* and *S. pneumoniae* (18). Furthermore, genomic analysis showed that DNA segments with homology to the Φ1207.3 SOS cassette are present in different streptococci and are carried on mobile genetic elements, which have the potential to spread the cassette. A recent work showed that the Streptococcal Mobilizable family of phages are widely distributed among different streptococcal species, carry antibiotic resistance and virulence genes associated with the SOS cassette and are mobilizable by a mechanism resembling conjugative transfer, possibly facilitated by the concomitant presence of ICEs ((64)). Here we demonstrated that Φ1207.3 also carries a functional SOS-like cassette conferring a transient hypermutable phenotype in response to stresses, which, under the selective pressure of antibacterial therapy, could result in the accumulation of mutations conferring additional antibiotic resistance phenotypes. The presence of the Φ1207.3 SOS-like cassette in *S. pneumoniae* confers a higher survival rate compared to the isogenic strains without prophage or carrying Φ1207.3Δ*orf6-orf11* devoid of the SOS-like cassette. On the one hand, the cells harbouring Φ1207.3 are capable of surviving when exposed to stresses, on the other hand Φ1207.3 ensures its survival within the lysogenic cell population. Further experiments will be needed to assess the effect of UV light exposure on prophage gene expression and production of phage particles. This would clarify whether phage replication or lysogenic conversion is favoured. The presence of erythromycin resistance genes, the ability to transfer among different bacterial species and the ability to confer a transient hypermutable phenotype in response to stresses highlight the clinical relevance of Φ1207.3.

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FIGURE LEGENDS

Figure 1. *S. pyogenes* Φ1207.3 SOS-like cassette. The prophage Φ1207.3 contains an SOS-like cassette located between the *mef*(A)-*msr*(D) gene pair conferring erythromycin resistance and the type II restriction-modification cassette constituted by *orf12*, *orf13* and *orf14*. The cassette contains 6 open reading frames (namely *orf6* to *orf11*) likely to be arranged in an operon. The *orf11* gene product is homologous to *L. lactis* HdiR LexA-like transcriptional repressor, Orf8 to the *S. pneumoniae* Tn*5253* UmuC/Orf70, Orf7 to the *B. subtilis* YolD-like protein, a putative functional homolog of UmuD. The putative promotor sequence of the SOS-like operon is indicated, ORFs and their direction of transcription are reported as arrows (in red *mef*(A)-*msr*(D), in green the SOS-like cassette, in blue the restriction-modification cassette).

Figure 2. Bacterial survival upon UV-C light irradiation. Effect of the Φ1207.3 SOS-like cassette on the *S. pneumoniae* bacterial survival upon UV-C light irradiation was investigated in absence of Φ1207.3 prophage (strain FR172, black circle), in presence of Φ1207.3 (FR173, green square) and in the presence of the recombinant Φ1207.3Δ*orf6-orf11*, lacking the SOS-like cassette (FR174, red triangle). Irradiation was carried out with UV-C light at wavelengths: A) 255 nm; B) 265 nm; C) 275 nm; D) 285 nm. No significant difference was observed between strain FR172, not carrying Φ1207.3, and FR174, carrying the recombinant Φ1207.3Δ*orf6-orf11* devoid of the SOS-like cassette. The presence of the Φ1207.3 in FR173 resulted in an increased bacterial survival compared to FR172, not carrying Φ1207.3, up to: i) 8.46-fold at 255 nm, ii) 29.11-fold at 265 nm, iii) 16.10-fold at 275 nm, iv) 7.26-fold at 285 nm, while compared to FR174, carrying the recombinant Φ1207.3ΔSOS-like cassette, up to i) 10.93-fold at 255 nm, ii) 34.21-fold at 265 nm, iii) 10.69-fold at 275 nm, iv) 8.23-fold at 285 nm. Results are reported as means of at least three independent experiments with standard deviations (error bars). In each experiment, 100% survival was based on the cell viability measured in plates not receiving UV-C light irradiation (0 J/m^2).

Figure 3. Mutation rates upon UV-C light irradiation.

Effect of the Φ1207.3 SOS-like cassette on the mutation rate in *S. pneumoniae* upon UV-C light irradiation was investigated in absence of Φ1207.3 prophage, in presence of Φ1207.3 and in the presence of the recombinant Φ1207.3ΔSOS-like cassette. Irradiation was carried out with UV-C light at wavelengths 255 nm, 265 nm, 275 nm, and 285 nm at a fluence of 50 J/m^2. Strain FR173, carrying Φ1207.3, showed a significant increase in the mutation rate (up to 18.38-fold at 285 nm) upon UV-C light treatment at every wavelength, whereas no significant increase was observed for strain FR172, not carrying Φ1207.3, and strain FR174, carrying the recombinant Φ1207.3ΔSOS-like cassette. The mutation rate was assessed by measuring the acquisition of rifampicin resistance. Results are reported as means of four independent experiments with 84% confidence intervals. *** adjusted *p*-value <0.001.

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537

538 **Table 1.** *S. pneumoniae* strains and relevant properties.

Strain	Relevant properties[a]	Origin (Reference)
R6	Unencapsulated transformation recipient, Hex^+	(Hoskins et al. 2001)
Rx1	Unencapsulated transformation recipient, Hex^-	(Cuppone et al. 2021)
FP10	Rx1 competence deficient derivate, Δ*comC*; *str-41*, Cm^R, Sm^R	(Santoro et al 2010)
FP11	Rx1 competence deficient derivate, Δ*comC*; *nov-1*, Cm^R, Nov^R	(Santoro et al 2010)
FR125	FP10 derivative carrying Φ1207.3; Δ*comC*, *str-41*, Δ*celB*; Cm^R, Sm^R, Spc^R, Em^R	(Iannelli unpublished)
FR3	FP10 derivative carrying Φ1207.3 (by transformation with FR125 chromosomal DNA), Cm^R, Sm^R, Em^R	(Iannelli unpublished)
FR169	FP11 derivative carrying Φ1207.3 (by conjugation with FR3), Cm^R, Nov^R, Em^R	(Iannelli unpublished)
FR170	FR3 derivative carrying Φ1207.3Δ*orf6-orf11* (by transformation with the *orf6-orf11* PCR mutagenic construct), Cm^R, Sm^R, Em^R, Km^R	(Iannelli unpublished)
FR171	FP11 derivative carrying Φ1207.3 Δ*orf6-orf11* (by conjugation with FR170), Cm^R, Nov^R, Em^R, Km^R Em^R	(Iannelli unpublished)
FR172	R6 derivative, *str-41* (by transformation with a PCR fragment obtained from FP10), Sm^R	This study
FR173	FR172 derivative carrying Φ1207.3 (by conjugation with FR169), Sm^R, Em^R	This study
FR174	FR172 derivative carrying Φ1207.3Δ*orf6-orf11* (by conjugation with FR171), Sm^R, Em^R, Km^R	This study

539

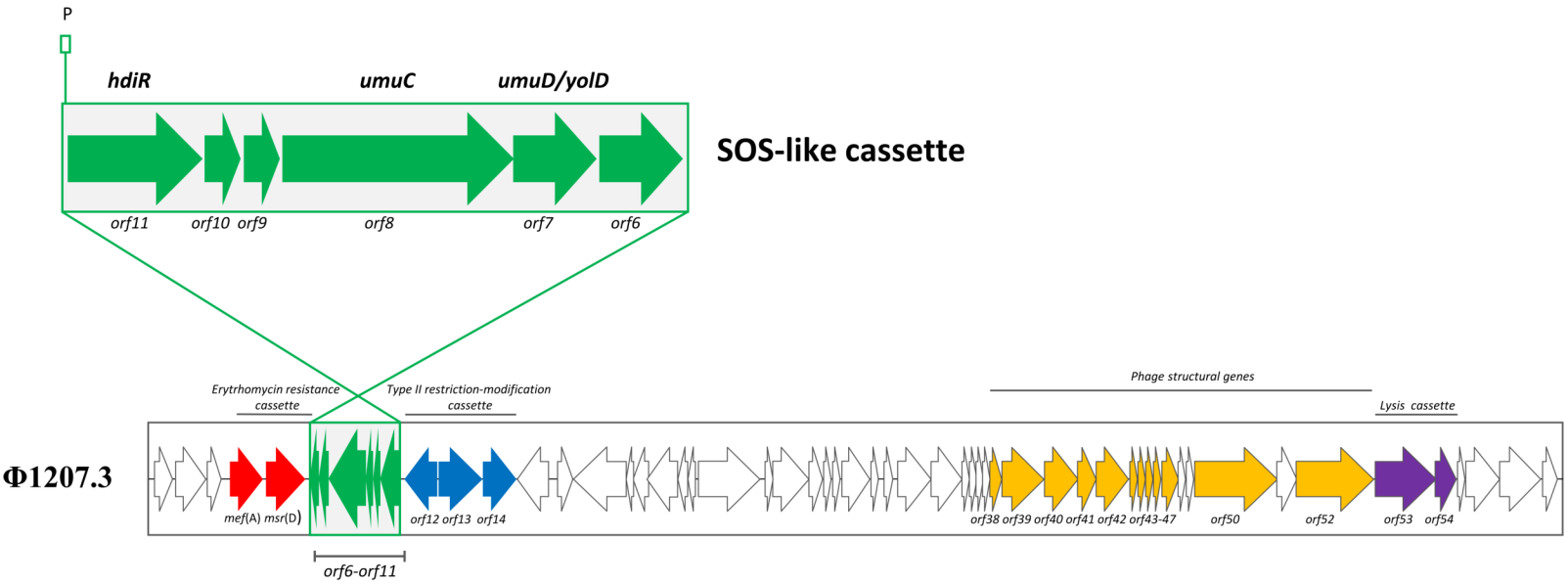
540 [a]Hex is the DNA Mismatch Repair System (65), *str-41* and *nov-1* indicate point mutations
541 conferring resistance to streptomycin and novobiocin, respectively (66, 67). Cm, chloramphenicol;

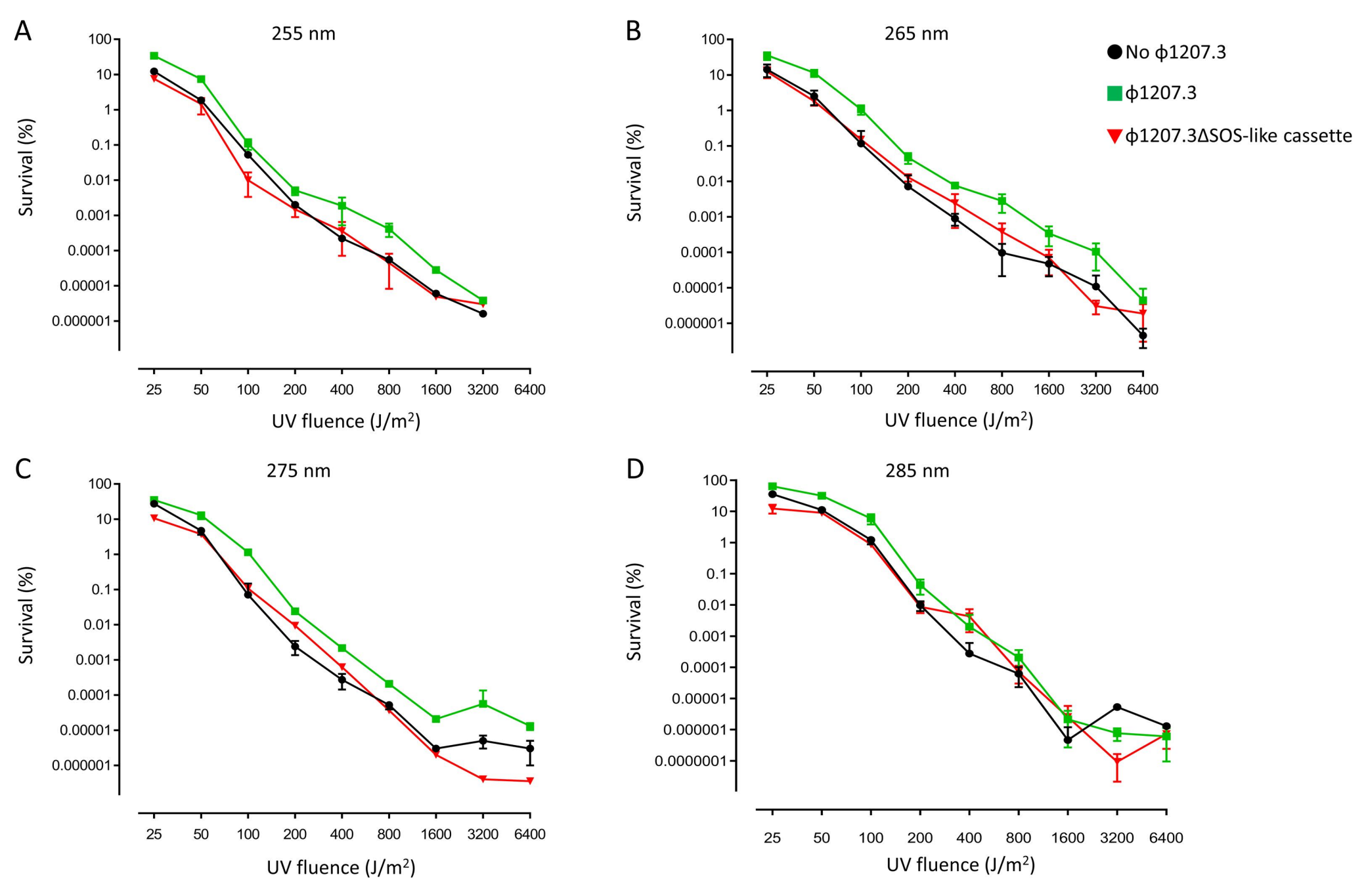
Sm, streptomycin; Nov, novobiocin; Spc, spectinomycin; Em, erythromycin; Km, kanamycin. The *orf6-orf11* region encodes the SOS-like cassette of the *S. pyogenes* Φ1207.3 prophage.

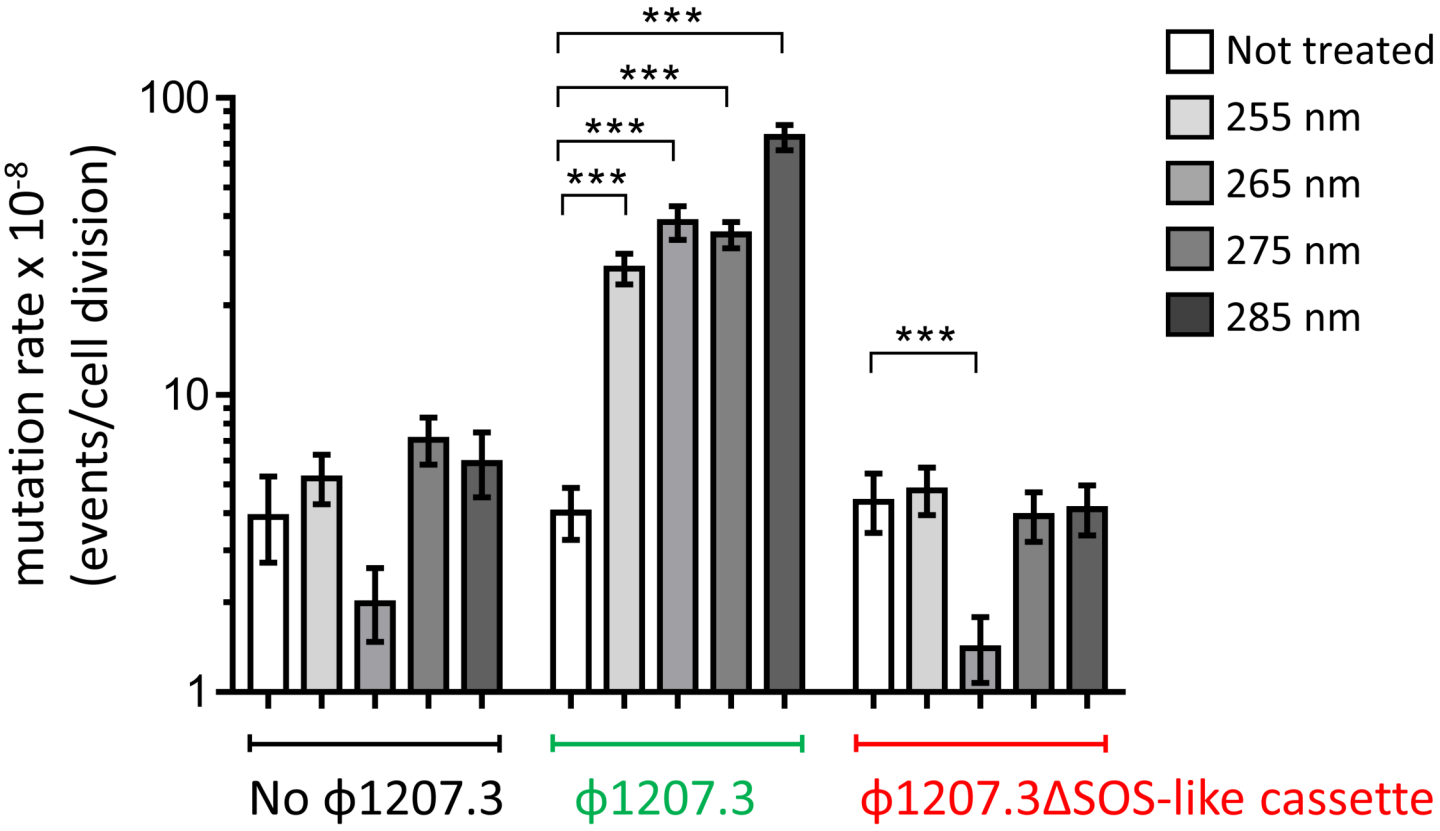
Table 2. Φ1207.3 SOS-like cassette predicted structural proteins.

ORF (aa)[a]	Predicted protein	Pfam domain(s) (aa)[b] [E value]	Conserved Domain Database (aa)[c] [E value]	Homologous protein ID / Origin (identity %) [E value][d]	Phyre2 (confidence)[e] [identity %]
orf6 (99)		DUF5960 (11-92) [2.0e-32]			Chain B lambda repressor, Escherichia virus Lambda (100%) [21%]
orf7 (122)	YolD-like protein	YolD-like protein (28–111) [3.2e-12]		WP_052006222.1/ *Bacillus subtilis* plasmid pLS20 (38) [2e-5]	Ribosome maturation factor rimp, *Escherichia coli* (88.9%) [16%]
orf8 (471)	SOS response UmuC-like protein	IMS (109-207) [2.3e-11] IMS_HHH (223-255) [4.5e-06] IMS_C (303-406) [4.9e-14]	PolY_Pol_V_umuC (15-400) [2.37e-121]	EU351020 orf70/Tn*5253* *Streptococcus pneumoniae* (73) [0.0]	Y-family DNA Polymerase, *Saccharolobus solfataricus* (100%) [25%]
orf9 (46)					Bacterial cell-division inhibitor MinC, *Thermotoga maritima* (30.4%) [36%]
orf10 (75)					Putative DNA mismatch repair protein, *Bacteroides thetaiotaomicron* (31.6%) [30%]
orf11 (229)	LexA-like repressor	HTH_3 (7-61) [1.1e-12] Peptidase_S24 (111-220)[4.3e-22]	HTH_XRE (6-61) [1.64e-14] Peptidase_S24 (111-220) [1.62e-29]	CAD89881.1/ *Lactococcus lactis subsp. cremoris* (31) [9e-32]	

[a] The number of the amino acid (aa) of the predicted protein is reported in parenthesis
[b] The number in parenthesis indicates the region of the protein homologous to the Pfam domain or to the domain presnt in the Conserved Domain Database
[c] Determined by compositional matrix adjustment
[e] Confidence indicates the reliability of the alignment


P
hdiR
umuC
umuD/yolD
orf11
orf10
orf9
orf8
orf7
orf6
SOS-like cassette
Erythromycin resistance cassette
Type II restriction-modification cassette
Phage structural genes
Lysis cassette
Φ1207.3
mef(A) msr(D)
orf12 orf13 orf14
orf38 orf39 orf40 orf41 orf42 orf43-47
orf50
orf52
orf53
orf54
orf6-orf11


A
255 nm
B
265 nm
C
275 nm
D
285 nm
No ϕ1207.3
ϕ1207.3
ϕ1207.3ΔSOS-like cassette
Survival (%)
UV fluence (J/m²)
100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
0.0000001
25
50
100
200
400
800
1600
3200
6400


mutation rate x 10-8
(events/cell division)
100
10
1
***
***
***
***
***
Not treated
255 nm
265 nm
275 nm
285 nm
No ϕ1207.3
ϕ1207.3
ϕ1207.3ΔSOS-like cassette