



The *mef(A)/msr(D)*-carrying streptococcal prophage Φ 1207.3 encodes an SOS-like system, induced by UV-C light, responsible for increased survival and increased mutation rate

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1 **The *mef(A)/msr(D)*-carrying streptococcal prophage Φ 1207.3 encodes**
2 **an SOS-like system, induced by UV-C light, responsible for increased**
3 **survival and increased mutation rate**

4

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22 mutation rate, bacterial survival, UV-C LED light, fluctuation test, DNA repair

23

24

25 **Abstract**

26 Bacterial SOS response is an inducible system of DNA repair and mutagenesis. Streptococci lack a
27 canonical SOS response, but an SOS-like response was reported in some species. The *mef(A)*-
28 *msr(D)*-carrying prophage Φ 1207.3 of *Streptococcus pyogenes* contains a region, spanning *orf6* to
29 *orf11*, showing homology to characterized streptococcal SOS-like cassettes. Genome-wide
30 homology search showed the presence of the whole Φ 1207.3 SOS-like cassette in three *S. pyogenes*
31 prophages, while parts of it were found in other bacterial species. To investigate whether this
32 cassette confers an SOS-mutagenesis phenotype, we constructed *Streptococcus pneumoniae* R6
33 isogenic derivative strains: (i) FR172, streptomycin resistant, (ii) FR173, carrying Φ 1207.3, and (iii)
34 FR174, carrying a recombinant Φ 1207.3, where the SOS-like cassette was deleted. These strains
35 were used in survival and mutation rate assays using a UV-C LED instrument for which we
36 designed and 3D-printed a customized equipment, constituted of an instrument support and
37 swappable-autoclavable mini-plates and lids. Upon exposure to UV fluences ranging from 0 to 6400
38 J/m² at four different wavelengths, 255, 265, 275 and 285 nm, we found that the presence of
39 Φ 1207.3 SOS-like cassette increases bacterial survival up to 34-fold. Mutation rate was determined
40 by measuring rifampicin resistance acquisition upon exposure to UV fluence of 50 J/m² at the four
41 wavelengths by fluctuation test. The presence of Φ 1207.3 SOS-like cassette resulted in a significant
42 increase in the mutation rate (up to 18-fold) at every wavelength. In conclusion, we demonstrated
43 that Φ 1207.3 carries a functional SOS-like cassette responsible for an increased survival and
44 increased mutation rate in *S. pneumoniae*.

45 **Importance**

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

51 we demonstrated that the SOS-like gene cassette of prophage Φ 1207.3 is functional, responsible for
52 a temporary hypermutable phenotype, and enhances bacterial survival to UV irradiation. Prophage
53 Φ 1207.3 also carries erythromycin resistance genes and can lysogenize different pathogenic
54 bacteria, constituting an example of a mobile genetic element which can confer multiple phenotypes
55 to its host.

56 **Introduction**

57 Bacteria evolve through chromosomal rearrangements, acquisition of DNA by horizontal gene
58 transfer and mutation. Mutations arise by a process referred to as mutagenesis which is measured
59 determining the mutation frequency or the mutation rate. Mutation frequency represents the
60 proportion of mutant cells in a specific culture, while the mutation rate is the probability of a
61 mutation occurring per cell division and thus is also an estimation of the probability of a mutation
62 occurring during the life of a bacterial cell (1–3). Mutation rates differ among bacterial species and
63 among strains of the same species, and are influenced by the fidelity of DNA replication, DNA
64 damage and repair pathways, and exposure to chemical or physical agents. In *Escherichia coli* DNA
65 replication is carried out by high fidelity polymerases which misinsert nucleotides at a rate of 10^{-4} -
66 10^{-6} per base pair (bp), but possess a 3'-exo proofreading activity that reduces mutation load by
67 about 100-fold (4). *E. coli* has also evolved the SOS response, which is inducible by DNA damage
68 and allows for improved survival and continuous replication at the cost of elevated mutagenesis (5,
69 6). The main proteins involved in SOS response are LexA, a homodimeric transcriptional repressor
70 of the SOS regulon, and RecA, which, upon DNA damage, binds to ssDNA, becomes activated, and
71 promotes the autocleavage of LexA and subsequent transcription of the SOS genes (7). The SOS
72 response induces the expression of genes involved in DNA-damage tolerance and repair, including
73 the Translesion Synthesis (TLS) system (8). TLS is involved in the tolerance to DNA damage, and
74 can be both error-free or error-prone, when the polymerase V is recruited. Polymerase V is encoded
75 by the *umuDC* operon, where *umuC* encodes a translesion DNA polymerase, of the Y family, while

76 *umuD* encodes a regulatory protein. The low fidelity polymerase V has a high rate of nucleotide
77 misincorporation (10^{-3} - 10^{-4} per bp) (9) and also lacks proofreading activity (10).
78 Streptococci lack a canonical SOS response (11), but SOS-like gene cassettes are present in the
79 genomes, either on the chromosome or on Mobile Genetic Elements, of *Streptococcus uberis*,
80 *Streptococcus agalactiae*, *Streptococcus pyogenes*, *Streptococcus sanguinis*, *Streptococcus mitis*,
81 and *Streptococcus thermophilus* (12). In *S. uberis*, the SOS-like cassette contains 4 genes arranged
82 in an operon including (i) *hdiR*, coding for a protein homologous to the HdiR regulator of
83 *Lactococcus lactis*, induced by heat shock and DNA-damage (13), (ii) *umuC* encoding an UmuC-
84 like protein, (iii) 2 genes of unknown function. The HdiR regulator of *S. uberis* undergoes
85 spontaneous self-cleavage at alkaline pH, similar to the RecA-mediated self-cleavage of LexA-like
86 proteins. HdiR specifically binds to an inverted repeat sequence downstream the *hdiR* putative
87 promoter, suggesting a role as transcriptional repressor of the SOS-like cassette. The pneumococcal
88 Integrative and Conjugative Element (ICE) Tn5253 confers UV resistance to the cell, inducing a
89 mutagenic SOS response (14). Tn5253 contains an SOS-like cassette similar to that described in *S.*
90 *uberis*, comprising: (i) *orf69*, which codes for an LexA-like repressor, (ii) *orf70*, which codes for an
91 UmuC-like protein, (iii) *orf71* and *orf72* of unknown function (15–17). In *S. pyogenes*, a gene
92 cassette homologous to the already known streptococcal SOS-like cassettes is associated with the
93 *mef(A)-msr(D)*-carrying prophages Φ 1207.3 (18–21), Φ 10394.4, Φ m46.1 and its variant
94 VP_00501.1 (22–25). Φ 1207.3 is a functional bacteriophage capable to lysogenize different strains
95 of the human pathogens *S. pyogenes* and *S. pneumoniae*, which colonize the upper respiratory tract
96 (26). Since both of these species can cause invasive infections, the transmission of antibiotic
97 resistance genes by lysogenic transfer of Φ 1207.3 assumes clinical relevance. In this work, we
98 transferred Φ 1207.3 prophage from the original *S. pyogenes* host to the *S. pneumoniae* standard
99 laboratory strain R6 to investigate if the SOS-like cassette carried by the Φ 1207.3 prophage is able
100 to activate an SOS-like response, which could increase the acquisition of mutations associated to
101 antimicrobial resistance.

102 **Materials and methods**

103 *Bioinformatic analysis*

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

113 *Bacterial strains and growth*

114 Streptococcal strains used in this work and their relevant properties are reported in Table 1. Strains
115 were grown in Tryptic Soy Broth (TSB, BD) at 37°C. Starter cultures were taken at an optical
116 density at 590 nm (OD₅₉₀) ranging from 0.2 to 0.3 and were frozen in 10% glycerol at -70°C. Solid
117 media were obtained by supplementing TSB with 1.5% agar (BD Difco) and 3% defibrinated horse
118 blood (Liofilchem). When required, both liquid and solid media were supplemented with antibiotics
119 at the following concentrations: 500 µg/ml kanamycin, 500 µg/ml streptomycin, 0.5 µg/ml
120 erythromycin, 5 µg/ml chloramphenicol and 20 µg/ml rifampicin.

121 *Lysogenization assays*

122 Transfer of bacteriophage Φ1207.3 was obtained through a plate mating experiment, as previously
123 described (28). Briefly, donor cells, carrying the phage, and recipient cells were grown separately in
124 TSB in the presence of the appropriate antibiotics. Upon reaching the end of the exponential phase,
125 cells were mixed at a donor-recipient 1:10 ratio, centrifuged, and the pellet was plated on TSA
126 plates supplemented with 5% horse blood. Plates were incubated at 37°C in the presence of 5% CO₂
127 for 4h, cells were then recovered with a cotton swab and resuspended in TSB/10% glycerol. To

128 select for lysogens, cell suspension was plated following a multilayer plating procedure in presence
129 of erythromycin and streptomycin which select for lysogens and counterselect the Φ 1207.3 donor
130 cells. To confirm the lysogens phenotype, a genetic analysis was performed as reported (16,28),
131 while PCR and direct Sanger sequencing of PCR template were used for genotype analysis
132 according to the protocol already described (32).

133 ***PCR Gene SOEing mutagenesis***

134 To generate a knock-out mutant of *orf6-orf11* region, PCR Gene Splicing by Overlap Extension
135 (SOEing) was used to produce a mutagenic construct containing the kanamycin resistance cassette
136 (876 bp) flanked by the DNA fragments located upstream of *orf6* (555 bp) and downstream of *orf11*
137 (829 bp) as previously described (29). The primer pair IF149/IF210 was used to amplify the 876-bp
138 *ami/aphIII* kanamycin resistance cassette (30), while IF242/IF239 and IF240/IF241 amplified the
139 flanking fragments. Assembly of the final construct (2,260 bp) was obtained with the IF242/IF241
140 primer pair. PCR was carried out as previously described (31). The PCR products were separated in
141 a 0.8% agarose gel at 200 V for 30 minutes, stained for 15 minutes in a 0.1% Ethidium Bromide
142 solution and visualized with UV light. PCR products after gel purification (NucleoSpin Gel and
143 PCR Clean-up kit, Macherey-Nagel) were quantified using the Qubit 2.0 Fluorometer (Thermo
144 Fisher Scientific). Oligonucleotide primers are listed in Table S1. The PCR mutagenic construct
145 was directly used as donor DNA in transformation experiments. , Since integration of Φ 1207.3 into
146 *S. pneumoniae* chromosome leads the disruption of the *celB* competence gene with a consequent
147 impairment of genetic transformation, we constructed transformable *S. pneumoniae* Rx1 derivative
148 strains carrying Φ 1207.3 integrated elsewhere in pneumococcal chromosome to be used as
149 transformation recipients (Table 1) (28). Pneumococcal competent cells were prepared as previously
150 described (29). Competent cells were thawed, supplemented with 25 ng/ml Competence Stimulating
151 Peptide (CSP) and 1 μ g/ml of the purified mutagenic construct, and the transformation mixture was
152 incubated at 37°C for 45 minutes. The resulting recombinant strains were selected for acquisition of
153 kanamycin resistance by multilayer plating (28). The deletion of the *orf6-11* region (nucleotides

154 6,189 to 9,297, GenBank accession no. [AY657002](#)) was confirmed by PCR and direct sequencing
155 of the PCR products (32).

156 *UV fluence measurement*

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

178 *Survival assay*

179 Bacterial survival upon UV-C light irradiation at different wavelengths (255 to 285 nm) was
180 assessed by plating the cells after exposure to different UV fluences from 0 to 6400 J/m² (0, 25, 50,
181 100, 200, 400, 800, 1600, 3200, 6400) and by calculating the cell viability for each strain. Cells
182 were grown at 37°C in 10 ml of TSB until reaching an OD₅₉₀ of 0.6 (about 1 x 10⁸ CFU/ml), when
183 they were centrifuged at 5,000 x g for 15 minutes, washed with sterile phosphate buffered saline
184 (PBS, Sigma Aldrich) and resuspended in 10 ml of PBS. UV-C survival experiments were carried
185 out by placing 1 ml of cells in the sterile Nylon-12 mini-plate and exposing them to different
186 fluences, under continuous stirring. UV-C irradiated and untreated control bacterial cells were
187 plated by multilayer plating. Survival was calculated as the ratio between UV-C irradiated and not
188 irradiated control cells.

189 ***Luria-Delbrück fluctuation test***

190 Mutation rate was determined by fluctuation analysis in absence or presence of UV-C light at
191 different wavelengths ranging from 255 to 285 nm. Strains were grown in TSB at 37°C until an
192 OD₅₉₀ of 0.6 and the number of total and rifampicin-resistant CFUs was determined prior to
193 freezing at -70°C in 1 ml-aliquots containing 10% glycerol. Rifampicin was used at a final
194 concentration of 20 µg/ml in the third layer of TSA, when appropriate (28). For mutation rate
195 determination, a set of 10 independent cultures was obtained by inoculating 5 ml of TSB with 1 x
196 10⁵ CFU of the same 1 ml frozen aliquot. For mutation rate determination following UV-C light
197 treatment, the 1 ml frozen aliquot was irradiated at an UV fluence of 50 J/m² before inoculum.
198 Then, to prepare 10 independent cultures, an inoculum corresponding to 1 x 10⁵ CFU was calculated
199 by inferring bacterial viability after irradiation from the data obtained with the UV-C light survival
200 assay. Cultures were grown at 37°C until they reached the mid-log phase, corresponding to about 12
201 generations, the number of total and rifampicin-resistant CFUs was determined by plating 0.1 ml of
202 culture and incubating the plates at 37°C for 48 h. For each strain and condition, 4 replicates of the
203 fluctuation test were carried out and the rifampicin-resistant CFUs numbers of the 40 cultures were
204 pooled into a single data set (33). The mutation rate determination and the statistical analysis were

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

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

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

220 **Results**

221 ***Φ1207.3 carries an SOS-like cassette***

222 The Φ 1207.3 DNA region located between the *mef(A)-msr(D)* erythromycin resistance cassette (20)
223 and the type II restriction-modification cassette (37), contains 6 open reading frames (namely *orf6*
224 to *orf11*) likely arranged in an operon (Figure 1). Homology search revealed that *orf11* predicted
225 gene product is homologous to the HdiR LexA-like transcriptional repressor of *Lactococcus lactis*,
226 *orf8* is homologous to the *Streptococcus pneumoniae* Tn5253 *umuC/orf70*, while *orf7* gene product
227 is homologous to the *Bacillus subtilis* Yold-like protein, which is predicted to be a functional
228 equivalent of UmuD (38) (Table 2). The deduced amino acid sequences of *orf6* to *orf11* share

229 homology (40-73%) with the predicted gene products of the *S. uberis* and of the *S. pneumoniae*
230 Tn5253 SOS-like cassettes, respectively (12, 14), with the exception of *orf9* and *orf10*, which
231 appear to be unique for Φ 1207.3. For these reasons, the *orf6* to *orf11* region of Φ 1207.3 was
232 referred to as an SOS-like cassette.

233 ***Presence of the Φ 1207.3 SOS-like cassette in bacterial genomes***

234 A genome-wide homology search of the Φ 1207.3 SOS-like cassette in the 67,343 complete
235 bacterial genomes available in the GenBank Microbial Genomes Database (accessed in March
236 2022) revealed the presence of the cassette, or parts of it, in 104 complete bacterial genomes (Figure
237 S2, Table S4). In 3 *S. pyogenes* genomes, a cassette 100% homologous to that of Φ 1207.3 was
238 found associated to *mef(A)-msr(D)*-carrying prophages, including Φ 10394.4. A copy of the SOS-
239 like cassette, containing a short deletion at the 5' end of *orf11*, was found in 12 additional
240 streptococcal genomes, including *Streptococcus gallolyticus*, *Streptococcus dysgalactiae*, and
241 *Streptococcus suis*. In the remaining 89 genomes, fragments of the cassette were found, including a
242 fragment spanning *orf6* to *orf9* in 6 *S. suis* genomes and a fragment spanning *orf6* to the partial 3'
243 end of *orf8* in 43 genomes, including 16 pneumococcal genomes. The fragment belongs to the
244 *mef(A)-msr(D)*-carrying element Tn1207.1 (39, 40) in the *S. pneumoniae* INV200 genome and to
245 the *mef(E)-msr(D)*-carrying mega element (41, 42) in the remaining 15 genomes.

246 ***Construction of isogenic *S. pneumoniae* recombinant strains***

247 To investigate if the presence of this cassette confers an SOS-mutagenesis phenotype we
248 constructed three isogenic derivative strains of the *S. pneumoniae* standard laboratory strain R6: (i)
249 FR172, streptomycin resistant derivative of R6, (ii) FR173, derivative of FR172 carrying Φ 1207.3,
250 and (iii) FR174, derivative of FR172 carrying a recombinant Φ 1207.3, where a 3109-bp DNA
251 fragment, corresponding to the *orf6* to *orf11* region, was deleted by substitution with a kanamycin
252 resistance cassette (Table 1). Isogenic strains were constructed in R6 since it harbours a functional
253 Mismatch Repair System (*hex+*) which results in a low basal mutation rate (43–46).

254 ***The presence of the Φ 1207.3 SOS-like cassette increases bacterial survival upon UV-C light***
255 ***irradiation***

256 To assess whether the SOS-like cassette of Φ 1207.3 is involved in increased survival upon UV-C
257 light treatment, the bacterial viability of the three isogenic strains was measured upon exposure to
258 UV fluences ranging from 0 to 6400 J/m² at four different wavelengths, 255, 265, 275 and 285 nm
259 (Figure 2). No significant difference in bacterial survival was observed between strain FR172, not
260 carrying Φ 1207.3, and FR174, carrying the recombinant Φ 1207.3 Δ orf6-orf11 devoid of the SOS-
261 like cassette. The presence of the Φ 1207.3 in FR173 increases bacterial survival upon UV-C
262 irradiation compared to FR172 and FR174, regardless of UV-C light fluences and wavelengths.
263 Noticeably, at a 6400 J/m² fluence we did not observe any increase in survival due to the SOS-like
264 cassette presence. It is likely that, at high UV fluences, the accumulation of DNA damages with the
265 associated high rate of mortality hinders the activation of the SOS-like response. FR173, carrying
266 Φ 1207.3, resulted in an increased bacterial survival compared to FR172, not carrying Φ 1207.3, up
267 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
268 nm. When compared to FR174, carrying the recombinant Φ 1207.3 Δ SOS-like cassette, FR173
269 resulted in an increased bacterial survival up to (i) 10.93-fold at 255 nm, (ii) 34.21-fold at 265 nm,
270 (iii) 10.69-fold at 275 nm, (iv) 8.23-fold at 285 nm (Table S5).

271 ***The Φ 1207.3 SOS-like cassette increases mutation rate upon UV-C light irradiation***

272 Activation of the Φ 1207.3 SOS-like response upon UV-C exposure was assessed determining the
273 mutation rate by measuring the acquisition of rifampicin resistance, associated to point mutations in
274 the *rpoB* gene coding for the β subunit of RNA polymerase (47, 48). A significant increase of
275 mutation rate was observed at every wavelength tested in FR173, carrying Φ 1207.3, whereas no
276 significant increase was observed in the FR172, not carrying Φ 1207.3, and FR174, carrying the
277 recombinant Φ 1207.3 Δ SOS-like cassette (Figure 3). In particular, in FR173 the mutation rate
278 increase of irradiated cells compared to not irradiated cells was: (i) 6.64-fold at 255 nm (p-value =
279 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

280 at 285 nm (p-value = 0), whereas in FR172 and FR174 no significant increase in mutation rate was
281 observed (Table S6). In absence of UV-C light irradiation, the three isogenic strains showed similar
282 mutation rate values, regardless of the presence of the Φ 1207.3 prophage or of the SOS-like
283 cassette.

284 **Discussion**

285 Mutation rate is generally low in bacteria, but hypermutable phenotype can arise as a consequence
286 of the loss of DNA proofreading during replication (Sundin and Weigand 2007; Gressel and Levy
287 2010; Jolivet-Gougeon et al. 2011). Stress conditions and DNA damage can also induce stress
288 response systems, which allow for improved survival and continuous replication at the cost of
289 elevated mutagenesis (6, 53). The SOS response is a DNA repair mechanism activated by some
290 bacteria in response to stressful conditions, which leads to a temporary hypermutable phenotype (5,
291 6). In the present work, we demonstrated that the *S. pyogenes* prophage Φ 1207.3 carries a
292 functional gene cassette responsible for the activation of an SOS-like response in *S. pneumoniae* in
293 response to UV-C light irradiation. We studied this cassette after its introduction in the *S.*
294 *pneumoniae* R6 strain, which contains a Mismatch Repair System (*hex*) conferring a low mutation
295 rate (43). We observed that the activation of this SOS-like cassette, upon UV-C exposure, makes
296 the R6 strain temporarily hypermutable, resulting in a higher ability of surviving and evolving.
297 Hypermutable strains have been found in pathogenic bacteria (49) and represent a huge problem in
298 clinical settings (54–57). Clinical strains are frequently exposed to variable and changing stressful
299 conditions, such as antibiotic treatments and host defenses, which can lead to evolution of resistance
300 and virulence phenotypes, with consequent therapeutic failures (33, 51, 58–62). Hypermutable
301 strains were also characterized in *S. pyogenes*, where the DNA Mismatch Repair (MMR) gene *mutL*
302 can be interrupted by the insertion of a genomic island. This genomic island is capable of growth-
303 phase-dependent excision and functions as a switch for the hypermutator phenotype (63). Prophage
304 Φ 1207.3, originally found in a clinical isolate of *S. pyogenes*, carries the erythromycin resistance
305 genes *mef(A)*-*msr(D)* (18–21) and is able to transfer among *S. pyogenes* strains with different

306 genetic background (unpublished data) and other streptococcal species such as *S. gordonii* and *S.*
307 *pneumoniae* (18). Furthermore, genomic analysis showed that DNA segments with homology to the
308 Φ 1207.3 SOS cassette are present in different streptococci and are carried on mobile genetic
309 elements, which have the potential to spread the cassette. A recent work showed that the
310 Streptococcal Mobilizable family of phages are widely distributed among different streptococcal
311 species, carry antibiotic resistance and virulence genes associated with the SOS cassette and are
312 mobilizable by a mechanism resembling conjugative transfer, possibly facilitated by the
313 concomitant presence of ICEs ((64)). Here we demonstrated that Φ 1207.3 also carries a functional
314 SOS-like cassette conferring a transient hypermutable phenotype in response to stresses, which,
315 under the selective pressure of antibacterial therapy, could result in the accumulation of mutations
316 conferring additional antibiotic resistance phenotypes. The presence of the Φ 1207.3 SOS-like
317 cassette in *S. pneumoniae* confers a higher survival rate compared to the isogenic strains without
318 prophage or carrying Φ 1207.3 Δ *orf6-orf11* devoid of the SOS-like cassette. On the one hand, the
319 cells harbouring Φ 1207.3 are capable of surviving when exposed to stresses, on the other hand
320 Φ 1207.3 ensures its survival within the lysogenic cell population. Further experiments will be
321 needed to assess the effect of UV light exposure on prophage gene expression and production of
322 phage particles. This would clarify whether phage replication or lysogenic conversion is favoured.
323 The presence of erythromycin resistance genes, the ability to transfer among different bacterial
324 species and the ability to confer a transient hypermutable phenotype in response to stresses
325 highlight the clinical relevance of Φ 1207.3.

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336 FIGURE LEGENDS

337 **Figure 1. *S. pyogenes* Φ1207.3 SOS-like cassette.** The prophage Φ1207.3 contains an SOS-like
338 cassette located between the *mef(A)-msr(D)* gene pair conferring erythromycin resistance and the
339 type II restriction-modification cassette constituted by *orf12*, *orf13* and *orf14*. The cassette contains
340 6 open reading frames (namely *orf6* to *orf11*) likely to be arranged in an operon. The *orf11* gene
341 product is homologous to *L. lactis* HdiR LexA-like transcriptional repressor, Orf8 to the *S.*
342 *pneumoniae* Tn5253 UmuC/Orf70, Orf7 to the *B. subtilis* Yold-like protein, a putative functional
343 homolog of UmuD. The putative promoter sequence of the SOS-like operon is indicated, ORFs and
344 their direction of transcription are reported as arrows (in red *mef(A)-msr(D)*, in green the SOS-like
345 cassette, in blue the restriction-modification cassette).

346 **Figure 2. Bacterial survival upon UV-C light irradiation.** Effect of the Φ1207.3 SOS-like
347 cassette on the *S. pneumoniae* bacterial survival upon UV-C light irradiation was investigated in
348 absence of Φ1207.3 prophage (strain FR172, black circle), in presence of Φ1207.3 (FR173, green
349 square) and in the presence of the recombinant Φ1207.3Δ*orf6-orf11*, lacking the SOS-like cassette
350 (FR174, red triangle). Irradiation was carried out with UV-C light at wavelengths: A) 255 nm; B)
351 265 nm; C) 275 nm; D) 285 nm. No significant difference was observed between strain FR172, not
352 carrying Φ1207.3, and FR174, carrying the recombinant Φ1207.3Δ*orf6-orf11* devoid of the SOS-
353 like cassette. The presence of the Φ1207.3 in FR173 resulted in an increased bacterial survival
354 compared to FR172, not carrying Φ1207.3, up to: i) 8.46-fold at 255 nm, ii) 29.11-fold at 265 nm,
355 iii) 16.10-fold at 275 nm, iv) 7.26-fold at 285 nm, while compared to FR174, carrying the
356 recombinant Φ1207.3ΔSOS-like cassette, up to i) 10.93-fold at 255 nm, ii) 34.21-fold at 265 nm,
357 iii) 10.69-fold at 275 nm, iv) 8.23-fold at 285 nm. Results are reported as means of at least three
358 independent experiments with standard deviations (error bars). In each experiment, 100% survival
359 was based on the cell viability measured in plates not receiving UV-C light irradiation (0 J/m²).

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

361 Effect of the Φ 1207.3 SOS-like cassette on the mutation rate in *S. pneumoniae* upon UV-C light
362 irradiation was investigated in absence of Φ 1207.3 prophage, in presence of Φ 1207.3 and in the
363 presence of the recombinant Φ 1207.3 Δ SOS-like cassette. Irradiation was carried out with UV-C
364 light at wavelengths 255 nm, 265 nm, 275 nm, and 285 nm at a fluence of 50 J/m². Strain FR173,
365 carrying Φ 1207.3, showed a significant increase in the mutation rate (up to 18.38-fold at 285 nm)
366 upon UV-C light treatment at every wavelength, whereas no significant increase was observed for
367 strain FR172, not carrying Φ 1207.3, and strain FR174, carrying the recombinant Φ 1207.3 Δ SOS-
368 like cassette. The mutation rate was assessed by measuring the acquisition of rifampicin resistance.
369 Results are reported as means of four independent experiments with 84% confidence intervals. ***
370 adjusted *p*-value <0.001.

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536

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 derivative, $\Delta comC$; <i>str-41</i> , Cm ^R , Sm ^R	(Santoro et al 2010)
FP11	Rx1 competence deficient derivative, $\Delta comC$; <i>nov-1</i> , Cm ^R , Nov ^R	(Santoro et al 2010)
FR125	FP10 derivative carrying $\Phi 1207.3$; $\Delta comC$, <i>str-41</i> , $\Delta celB$; Cm ^R , Sm ^R , Spc ^R , Em ^R	(Iannelli unpublished)
FR3	FP10 derivative carrying $\Phi 1207.3$ (by transformation with FR125 chromosomal DNA), Cm ^R , Sm ^R , Em ^R	(Iannelli unpublished)
FR169	FP11 derivative carrying $\Phi 1207.3$ (by conjugation with FR3), Cm ^R , Nov ^R , Em ^R	(Iannelli unpublished)
FR170	FR3 derivative carrying $\Phi 1207.3\Delta orf6-orf11$ (by transformation with the <i>orf6-orf11</i> PCR mutagenic construct), Cm ^R , Sm ^R , Em ^R , Km ^R	(Iannelli unpublished)
FR171	FP11 derivative carrying $\Phi 1207.3\Delta orf6-orf11$ (by conjugation with FR170), Cm ^R , Nov ^R , Em ^R , Km ^R Em ^R	(Iannelli unpublished)
FR172	R6 derivative, <i>str-41</i> (by transformation with a PCR fragment obtained from FP10), Sm ^R	This study
FR173	FR172 derivative carrying $\Phi 1207.3$ (by conjugation with FR169), Sm ^R , Em ^R	This study
FR174	FR172 derivative carrying $\Phi 1207.3\Delta orf6-orf11$ (by conjugation with FR171), Sm ^R , Em ^R , Km ^R	This study

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

542 Sm, streptomycin; Nov, novobiocin; Spc, spectinomycin; Em, erythromycin; Km, kanamycin. The
543 *orf6-orf11* region encodes the SOS-like cassette of the *S. pyogenes* Φ 1207.3 prophage.

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545

Table 2. Φ 1207.3 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 %]
<i>orf6</i> (99)		DUF5960 (11-92) [2.0e-32]			Chain B lambda repressor, Escherichia virus Lambda (100%) [21%]
<i>orf7</i> (122)	YolD-like protein	YolD-like protein (28-111) [3.2e-12]		WP_052006222.1/ <i>Bacillus subtilis</i> plasmid pLS20 (38) [2e-5]	Ribosome maturation factor rimP, <i>Escherichia coli</i> (88.9%) [16%]
<i>orf8</i> (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 <i>orf70/Tn5253</i> <i>Streptococcus</i> <i>pneumoniae</i> (73) [0.0]	Y-family DNA Polymerase, <i>Saccharolobus solfataricus</i> (100%) [25%]
<i>orf9</i> (46)					Bacterial cell-division inhibitor MinC, <i>Thermotoga maritima</i> (30.4%) [36%]
<i>orf10</i> (75)					Putative DNA mismatch repair protein, <i>Bacteroides</i> <i>thetaiotaomicron</i> (31.6%) [30%]
<i>orf11</i> (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/ <i>Lactococcus lactis</i> <i>subsp. cremoris</i> (31) [9e-32]	

^aThe number of the amino acid (aa) of the predicted protein is reported in parenthesis

^bThe number in parenthesis indicates the region of the protein homologous to the Pfam domain or to the domain present in the Conserved Domain Database

^cTerminated by compositional matrix adjustment

^dConfidence indicates the reliability of the alignment

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