Non-canonical *Staphylococcus aureus* pathogenicity island repression

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ABSTRACT

Mobile genetic elements control their life cycles by the expression of a master repressor, whose function must be disabled to allow the spread of these elements in nature. Here, we describe an unprecedented repression-derepression mechanism involved in the transfer of *Staphylococcus aureus* pathogenicity islands (SaPIs). Contrary to the classical phage and SaPI repressors, which are dimers, the SaPI1 repressor StlSaPI1 presents a unique tetrameric conformation never seen before. Importantly, not just one but two tetramers are required for SaPI1 repression, which increases the novelty of the system. To derepress SaPI1, the phage-encoded protein Sri binds to and induces a conformational change in the DNA binding domains of StlSaPI1, preventing the binding of the repressor to its cognate StlSaPI1 sites. Finally, our findings demonstrate that this system is not exclusive to SaPI1 but widespread in nature. Overall, our results characterize a novel repression-induction system involved in the transfer of MGE-encoded virulence factors in nature.

INTRODUCTION

*Staphylococcus aureus* pathogenicity islands (SaPIs) are prototypical members of a widespread family of mobile genetic elements (MGEs), the phage-inducible chromosomal islands (PICIs) (1). SaPIs are clinically important because they encode and disseminate toxin and antibiotic resistance genes (1,2). Normally, these elements reside passively integrated in host bacterial chromosomes under the control of a master repressor protein, Stl (SaPI transcription leftward) (3). Unlike most phage repressors, SaPI Stl repressors are not cleaved after induction of the cellular SOS response. This is because the Stl repressors are insensitive to the activated RecA protein. Instead, SaPI activation depends on the formation of a complex between Stl and specific phage proteins, which act as inducers of the SaPI life cycle (4–6). Since SaPIs require the phage components for DNA packaging and particle assembly (7,8), this strategy ensures that SaPIs will be induced only in the presence of their prey, the phages.

Different SaPIs encode different repressors and therefore require different phage proteins as inducers. Thus, the inducers for SaPIbov1, SaPI1 or SaPI2 are the phage-encoded dUTPases, Sri or recombinase proteins, respectively (4–6). To gain more insight into this interesting and distinctive induction-repression mechanism of the SaPIs, we recently solved the structure of the SaPIbov1 Stl (StlSaPIbov1) repressor alone and complexed with two different inducing proteins: the dimeric and trimeric dUTPase proteins of phages O11 and ϕ11, respectively (9). Our studies revealed that StlSaPIbov1 is a canonical dimer, with a modular structural organization reminiscent of many well-studied phage and MGE repressors, including the CI repressor of archetypic

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cal phage λ (9,10). These repressors have N-terminal domains that recognize and bind to their cognate DNA operator regions, and C-terminal domains that are involved in repressor dimerization and inducer recognition. Phages and other MGEs that are activated by the SOS response encode repressors that are structural homologs of LexA and are induced to undergo self-cleavage by activated RecA*. Since repressor dimers are required for repression, self-cleavage disrupts dimerization and activates the life cycle of prophages and other MGEs (11,12). In the case of SaPI1 bov1, StlSaPI1 forms dimers that are disrupted upon binding to their cognate phage inducers (9).

Our previous findings suggested that the general mechanism for the inactivation of StlSaPI1 and the classical CI-like repressors was disruption of their dimerization, though the ways by which this occurred were different. In the case of StlSaPI1, a domain with the dual role of mediating dimerization and binding inducer enables the SaPI1 bov1 island to sense the activation of a helper phage. However, the existence of multiple different Stl repressor proteins raises the question of whether the StlSaPI1 mechanism of inactivation is conserved across all SaPI repressors or whether they sense helper phages via other unknown strategies. To answer this question, we analysed SaPI1, which is one of the prototype typical islands used to decipher the biology of the SaPIs. It is also clinically relevant because it encodes TSST-1, the toxin responsible for a rare but important human disease known as toxic shock syndrome (13). Two additional factors reinforced the use of SaPI1 as a model. Firstly, Sri, its anti-repressor protein (5), is a small protein of 6.2 kDa, which reinforces the use of SaPI1 as a model. Secondly, and contrary to what is seen with SaPI-1, the ways by which this occurred were different. In the case of SaPI1 bov1, StlSaPI1 forms dimers that are disrupted upon binding to their cognate phage inducers (9).

Since repressor dimers are required for repression, self-cleavage disrupts dimerization and activates the life cycle of prophages and other MGEs that are activated by the SOS response (9,10). These repressors have N-terminal domains that recognize and bind to their cognate DNA operator regions, and C-terminal domains that are involved in repressor dimerization and inducer recognition. Phages and other MGEs that are activated by the SOS response encode repressors that are structural homologs of LexA and are induced to undergo self-cleavage by activated RecA*.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Supplementary Table S1. Strains were grown at 37°C in Luria-Bertani broth agar or in Luria-Bertani broth with shaking (180 rpm) for Escherichia coli, or in tryptic soy (TSA) agar or TSB broth for S. aureus. Ampicillin (100 mg/ml) or tetracycline (20 mg/ml; all Sigma-Aldrich) antibiotics were added when appropriate.

Plasmid construction

The plasmids used in this study (Supplementary Table S2) were constructed by cloning with T4 ligase (Thermofisher) the PCR products, amplified with the oligonucleotides listed in Supplementary Table S3 (Sigma-Aldrich), into the appropriate vectors after their digestion with restriction enzymes. The cloned plasmids were verified by Sanger sequencing (Eurofins Genomics). StlSaPI1 (Uniprot Accession code O54475) and 80α phage Sri (Uniprot Accession code A4ZF88) proteins were cloned into the pPROEX Hta plasmid. For StlSaPI1 expression alone, a His6-tag and TEV protease cleavage site were added to the N-terminal part of the protein. For the Sri-StlSaPI1 complex expression, both proteins were cloned into plasmid pPROEX Hta, under the control of the Trc IPTG inducible promoter, but only the Sri protein had the His6-tag and the TEV protease cleavage site fused to its N-terminal region. In this plasmid expressing both proteins, an extra ribosomal binding site was added upstream to the SaPI1 stl gene (3’ of the sri gene). For the in vivo experiments, the SaPI1 region between int and xis, which includes the entK, entQ, stl and str genes and the putative Stl binding sites present in the intergenic region between stl and str, was cloned into the plasmids pCN41 with the β-lactamase gene fused to xis.

Protein expression and purification

Proteins were overexpressed from E. coli BL21(DE3) (Novagen) cells transformed with the corresponding expression plasmids (Supplementary Table S2). Cultures were grown in Luria-Bertani broth to an OD600 of 0.5–0.6 and protein expression was induced with 1 mM IPTG at 20°C for 16 h. Cells were harvested by centrifugation at 4°C, 4000 rpm for 20 min, resuspended in lysis buffer (100 mM Tris pH 8, 300 mM NaCl, 5 mM MgCl2, 1 mM β-mercaptoethanol and protease inhibitor tablets (complete tablets, Roche) and lysed by sonication. The soluble fractions were obtained by centrifugation at 4°C, 15 000 rpm for 30 min and loaded onto a pre-equilibrated Nickel affinity column (Histrap 1 ml; GEHealthcare). After washing the column with a buffer with 20 mM imidazole, the proteins were eluted with a lysin buffer containing 300 mM imidazole. The fractions containing the eluted proteins were further purified by size exclusion chromatography using a Superdex S200 16/600 column and analysed by SDS-PAGE. Fractions containing the purest protein were selected, concentrated to 8–10 mg/ml and stored at –80°C.

For protein crystallization, the Sri-StlSaPI1 selenomethionine-labeled (SeMet) derivative complex was obtained using SelenoMethionine Medium Complete (Molecular Dimensions Ltd; MD 12–500), according to the manufacturer instructions, and purified as described previously. The Sri-StlSaPI1 and Sri-StlSaPI1 SeMet complexes used for crystallization, SEC-MALS and biolayer interferometry were digested with the TEV protease to eliminate the His6-tag at 4°C overnight in 100 mM Tris–HCl pH 8.5, 300 mM NaCl and 5 mM Mg2Cl buffer before further purification by size exclusion.

Protein crystallization and data collection

Crystals of 80α Sri-StlSaPI1 SeMet derivative and 80α Sri-StlSaPI1 L201E complexes were obtained by vapor-diffusion technique using a sitting drop setup at 15°C in a reservoir solution of 0.4 M ammonium phosphate, 25% PEG200 or 0.1 M Tris–HCl pH 8.5, 22% PEG350, respectively. The crystals were cryo-protected using 30% PEG200 or
Table 1. Data collection and refinement statistics. Data collection and refinement statistics for Sri-StlSaPI1 and Sri-StlSaPI1 L201E X-ray structures. PDB, protein data bank. Highest-resolution shell details are shown in parenthesis.

|                  | 80a Sri-StlSaPI1 | 80a Sri-StlSaPI1 L201E |
|------------------|------------------|-----------------------|
| **Data collection** |                  |                       |
| Space group      | P6_1 22          | P3_21                 |
| Cell dimensions a, b, c (Å) | 100.21, 100.21, 88.22 | 88.22, 88.22, 110.49 |
| α, β, γ (°)      | 90, 90, 120      | 90, 90, 120           |
| Wavelength (Å)   | 0.979            | 0.916                 |
| Resolution       | 86.79–2.90       | 62.84–2.97            |
| (3.08–2.99)      | (3.17–2.97)      |                       |
| Rmerge (%)       | 0.072 (0.514)    | 0.054 (0.589)         |
| Mean I/σ(I)      | 14.2 (2.4)       | 12.3 (1.3)            |
| Completeness (%) | 99.7 (99.5)      | 92.0 (46.3)           |
| Multiplicity     | 5.9 (5.8)        | 9.8 (9.3)             |
| Rwork/Rfree (%)  | 0.041 (0.246)    | 0.046 (0.629)         |
| Number of atoms: |                  |                       |
| Protein          | 4208             | 104.87                |
| Water            | 8                | 104.38                |
| B-factors (Å)    | 104.87           | 73.8                  |
| r.m.sdeviation   | 0.0050           | 0.0018                |
| Bond angles (°)  | 1.32             | 1.15                  |
| **PDB code**     | 7PA4             | 1ZVI                  |

PEG350 solution for Sri-StlSaPI1 or Sri-StlSaPI1 L201E respectively when freezing in liquid nitrogen. Sri-StlSaPI1 single-wavelength anomalous diffraction (SAD) was undertaken on beamline I03 at the Diamond Light Source synchrotron radiation facility (DLS; Didcot, UK) (15) at a wavelength of 0.98 Å. The Sri-StlSaPI1 L201E native dataset was collected on beamline I04-1 at DLS at a wavelength of 0.915 Å. For Sri-StlSaPI1 SeMet, data were indexed, integrated, and anisotropically scaled using the program DIALS and phasing was performed with CRANK2 both from CCP4 suite (17). The anomalous map for Sri-StlSaPI1 data set was obtained with Phenix suite and the peak intensities at the coordinates of selenium atoms in methionine residues are collected in Supplementary Table S4. For Sri-StlSaPI1 L201E, data were indexed, integrated, and scaled using the program AutoProc (16) and the phasing was performed by Molecular Replacement with Phaser (17) using the monomer of wt Sri-StlSaPI1 complex as a search model. Final models were generated by several rounds of manual model building using Coot (18) and computational refinement with Refmac5 (17). The crystallographic parameters, data-collection and refinement statistics are listed in Table 1.

**Size-exclusion chromatography multi-angle light scattering (SEC-MALS)**

The wt and L201E StlSaPI1 proteins at 2 mg/ml alone or in complex with Sri in 50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM MgCl2 were loaded in a Protein KW403 column (Shodex) equilibrated with the same buffer using a HPLC (Shimadzu) system. Chromatography was run at 0.4 ml/min and the UV, light scattering and differential refractive index (dRI) was monitored using a TREOS (Wyatt) system. The data collection and analysis were performed using ASTRA 7.3.2.21 software. The UV and MW representation was done by Excel.

**Characterization of the SaPI1 str and stl promoters**

To characterize the str and stl promoters, RNA extraction using an Ambion kit (Novartis) was performed according to the manufacturer instructions using the RN4220 strain lysogenic for 80a carrying SaPI1, 90 min after prophage induction with 2 µg/ml mitomycin C (MC). A 5’/3’ RACE Kit (Roche) was used to amplify the RNA obtained and the final DNA was sequenced to obtain the transcription start site nucleotide of both promoters. The primers used are summarised in the Supplementary Table S5. The –10 and –35 RNA pol binding sites were localized after the analysis of the DNA sequence.

**β-Lactamase assays**

Cells were obtained at different time points after mitomycin C (MC) induction of lysogenic strains carrying the appropriate plasmids. β-lactamase assays, using nitrocefin as substrate, were performed as described (4,6). Briefly, 50 µl of the collected sample were mixed with 50 µl of nitrocefin stock solution (192 µM made in 50 mM potassium phosphate buffer, pH 5.9), and the absorbance at 490 nm immediately read using a FLUOstar Omega microplate reader (BMG LABTECH) for 45 min. Promoter activity was calculated as Promoter activity = (Å540) / × 1,000, where Å540 is the absorbance of the sample at 540 nm at collection t, t is time, d is the dilution factor, and V is the sample volume.

**End-labeling SaPI1 stl-str DNA for footprinting experiments**

SaPI1 stl-str DNA was amplified using PfuTurbo DNA polymerase (Agilent), dNTPs (Invitrogen), and primers GC83016C (5’-GTTCATTTAACCAGAAGCTTCAAC TCACTTTTTC-3’) and GC83016B (5’-CAGTACC GTCGTGATAACGATGATTGATGTGATTT-3’). The PCR product was diluted 1:1,000 (approximately 0.001 pmol/µl) for preparing radiolabeled SaPI1 stl-str DNA. 5 pmol of primer (5 µl of 5 µM stocks) were end-labeled with 10 units of USB Optikinase (Affymetrix), and 7 µl of 6000Cu/mmol ATP-γS2P (Perkin-Elmer) in 25 µl total volume. Incorporation of ATP-γS2P into primer was determined by TCA precipitation of a 5 µl aliquot of a 1:200 dilution and counting the precipitated material on filter paper using a scintillation counter. To obtain final radiolabelled SaPI1 stl-str DNA (*) used in footprint experiments, the remaining 24.5 µl of the undiluted Optikinase reaction (containing ~1 pmol/5 µl of either top- or bottom-strand labelled primer) was added to the original 1:1000 dilution of the SaPI1 PCR product (template), 1 µl of 10 mM dNTP (Invitrogen), and 1 µl of PfuTurbo DNA polymerase (Agilent) for amplification in a final volume
of 50 μL. Unincorporated primers were removed using a QIAquick PCR Purification Kit (Qiagen) and eluted twice with 30 μL of water. To assess the final concentration of SaPI1 stl-str DNA*, a 1:200 dilution of the PCR clean-up material was made and dpm assessed using the same TCA precipitation/filter method and scintillation counter.

**G/A ladder of SaPI1 stl-str DNA**

The G/A ladder was generated using the piperidine method. First, 12 μL of SaPI1 stl-str DNA* was added to 2 μL of 1 mg/ml salmon sperm DNA and 8 μL of tris-EDTA (TE), pH 8 and incubated on ice. Second, 2 μL of 4% formic acid made fresh from stock was added and incubated at 37°C for 45 min, and subsequently placed back on ice. Third, 300 μL of piperidine (Sigma-Aldrich) was added and incubated at 90°C for 30 min, and subsequently placed back on ice. Fourth, 10 μL of 5 mg/ml salmon sperm DNA (50 μg total) was added followed by gentle mixing. Fifth, 1 mL of butanol was added, mixed, and centrifuged at ~20 000 rcf (g) for five min; the top layer was carefully removed. A further 1.2 mL of butanol was added, mixed, and centrifuged resulting in a pellet that contained precipitated DNA. Supernatant was removed and the pellet was gently washed with 150 μL of 1% SDS, followed by butanol precipitation (one 1 mL, and two 0.5 mL butanol precipitations). The DNA pellet was dehydrated with a speed vacuum, and resuspended in 20 μL of footprint loading buffer (0.5 mL deionized formamide; 20 μL of 0.25M EDTA, pH 7, 5 μL XCFF and 5 μL of BPE for visualization of sample migration during electrophoresis) and stored at ~20°C. Lastly, 1:7.5 and 1:15 dilutions of the G/A ladder were made using the same footprint loading buffer in order to obtain exposure intensities more suitable for sequence determination of the Stl footprints on SaPI1 stl-str DNA.

**DNase I footprinting**

Footprinting reactions were carried out in EMSA buffer (PBS, pH 7.3, 75 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, 5% glycerol) supplemented with 2 mM CaCl₂, which is required for Dnase I activity, and 100 ng/μL poly(d[I-C]) (Sigma-Aldrich), which helps reduce nonspecific protein interactions with nucleic acids. The final volume of each footprint reaction was 11 μL. First, a master mix of SaPI1 stl-str DNA was made as follows (per 10 total reactions); 10 μL of 0.5 pmol/μL SaPI1 stl-str DNA, 10 μL of 1 μg/μL poly(d[I-C]), 20 μL of 5× EMSA buffer, and 2 μL of 100 mM CaCl₂. 4 μL of SaPI1 stl-str DNA master mix was aliquoted into individual tubes on ice. 6 μL of ~3.3× Stl and/or Sri dilutions were made in EMSA buffer, pre-incubated on ice for 30 min, then added to 4 μL of SaPI1 stl-str DNA master mix, and allowed to incubate on ice for a further 30 min. To initiate DNase digestion, 1 μL of DNaseI (2 U/μL) was added to individual reactions and immediately transferred to a 37°C water bath for 10 min. Reactions were quenched by adding 17.2 μL of footprint loading buffer (0.5 mL deionized formamide; 20 μL of 0.25 M EDTA, pH 7; 5 μL XCFF and 5 μL of BPE), followed by immediate transfer to dry ice. Samples were boiled at 100°C for 2 min immediately before loading onto a denaturing gel (5% polyacrylamide, 7 M urea).

To help to increase the resolution of the gel electrophoresis, denaturing gels were pre-electrophoresed in 0.5× TBE before adding samples (500 V/400 mA/400 W for 30 min, then 750 V/400 mA/400 W for 30 min; then 1000 V/400 mA/400 W for 30 min and 1250 V/400 mA/400 W for >30 min). 15 μL of the quenched footprint reactions and the G/A ladder dilutions (discussed above) were loaded onto gels. After samples were added to the pre-electrophoresed gel, electrophoresis was continued for ~3500 Vh for optimal resolution of the Stl-bound regions in SaPI1 stl-str DNA. G + A sequencing reactions were performed on the same end-labeled fragments using the method of Maxam and Gilbert (19) and run on the same gel to identify the sequences protected by Stl.

**Electrophoretic mobility shift assay (EMSA)**

The StlSaPI1 DNA binding regions for the EMSA experiments were obtained commercially and hybridized at 95°C for 15 min with equal concentrations of the forward and reverse primers (Supplementary Table S6). DNA at a final concentration of 1 μM and increasing concentrations of the wt or the L201E mutant StlSaPI1 proteins (0.5–8 μM) were mixed with 1 μg/ml poly(d[I-C]) (Roche) in EMSA buffer (50 mM Tris–HCl pH 8, 5 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA and 5% glycerol) and incubated for 30 min at room temperature. The samples were then loaded onto 6% Tris-borate–EDTA (TBE) polyacrylamide gels and were electrophoresed in TBE buffer at 90 V for 1–2 h. Gels were stained with Gel Red (Biotium) for 10 min in shaking conditions and analysed with a ChemiDoc imaging system (BioRad).

**Biolayer interferometry**

The DNA binding kinetics assays with StlSaPI1 and StlSaPI1 L201E were performed by Biolayer Interferometry with an Octet RED96s System (Sartorius) in 50 mM Tris–HCl pH 8, 300 mM NaCl, 5mM MgCl₂, 50 μM EDTA pH 8, 0.005% TWEEN at 25°C and agitation speed of 1400 rpm. The 5´ biotinylated DNA probes, see Supplementary Table S6 for primers sequences, were immobilized at 1.562 μg/ml on previously hydrated streptavidin (SA) Biosensors (Sartorius) for 120 s, reaching a signal of ~2 nm. After DNA loading, the sensors were washed for 60 s in buffer for a stable baseline and protein association was monitored for 160 s using protein concentrations in a range between 1.562 and 100 nM. The protein concentration range was obtained by 1:2 serial dilutions of the initial stock. After that, dissociation in the same buffer was monitored for 200 s. An empty sensor was used as signal drift control and such signal was subtracted from the obtained curves. The association and dissociation constants (Kₐ and K₅) were obtained by fitting a 1:1 model for StlSaPI1 L201E and a mass transport model for StlSaPI1 in Octet BLI Discovery 12.2.2.20 software.

**Pull-down experiments**

The wild type (wt) and the Y76A versions of the StlSaPI1-Sri complex were expressed in 20 ml of culture as previously
indicated. The cells where then lysed with BugBuster protein extraction reagent (Novagen) for 30 min at room temperature. The soluble fraction was then incubated for 1 h with HisPur Ni-NTA resin (Thermofisher), and the proteins bound to the resin purified as described before. After purification, the fractions were analysed by SDS-PAGE, stained with Instant Blue (Expedeon) and visualized with a ChemiDoc imaging system (BioRad).

**SaPI transfer**

*S. aureus* strains lysogenic for 80α phage and containing SaPI1 were grown to early exponential phase (OD500 ~ 0.15) at 37°C and 120 rpm. Cultures were then induced by the addition of MC (2 μg/ml) and incubated for 4–5 h at 30°C followed by overnight incubation at room temperature before filtering the lysate with a 0.2 μm syringe filter. For SaPI titre determination, *S. aureus* RN4220 strain was grown overnight at 37°C and 120 rpm. The culture OD was adjusted to OD500 ~ 1.4 with TSB and supplemented with 4.4 mM CaCl2. 100 μl of the appropriate lysate dilution were added to 1 ml of this cell suspension and incubated for 30 min at 37°C. Three ml of transduction top agar (TTA, 30 g/l TSB, 7.5 g/l agar) were added to the transduction and the mix poured onto a TSA plate containing the appropriate antibiotic. Plates were incubated for 16–24 h at 37°C prior to determination of transducing units.

**Size-exclusion chromatography small angle X-ray scattering (SEC-SAXS)**

SEC-SAXS was done on beamline B21 of the Diamond Light Source synchrotron (Didcot, UK). Data were recorded at 12.4 keV, at a sample-detector distance of 4.014 m using a Pilatus 2 M detector (Dectris, Switzerland). 50 μl of protein samples at concentrations of 10.0 mg/ml (StlSaPI1) and 6.5 mg/ml (StlSaPI1–Sri) were loaded onto a Superdex 200 Increase 3.2 size exclusion chromatography column in 10 mM Tris pH 8, 1% (w/v) sucrose at 0.075 ml/min using an Agilent 1200 HPLC system. The column outlet was fed into the experimental cell, and 620 × 3.0 s frames of SAXS data were recorded. Data were processed with Sc变速箱 IV (http://www.bioisis.net) as follows. The integral of ratio to background signal along with the estimated radius of gyration (Rg) for each frame was plotted. Frames within regions of low signal and low Rg recorded prior to protein elution were selected as buffer and subtracted from frames within regions of higher signal and constant Rg. Subsequent analysis was performed using the ATSAS 3.0 suite of programs (20). The radius of gyration Rg was obtained from the Guinier approximation following standard procedures. The pairwise distance distribution function p(r) was computed using the indirect Fourier transformation method implemented in GNOM (21). From the p(r) function, an alternative estimate of Rg and the maximum particle dimension Dmax were obtained. Molecular weights were estimated by Bayesian inference (22) in Prinmus (20). Ensemble optimization modelling was undertaken with EOM (23). 10 000 models were generated in which the C-terminal domains (CTD, residues 101–247) were kept in the conformation observed by X-ray crystallography but the DBDs (residues 1–89) allowed to adopt positions consistent with their connection to the CTD via a native-like flexible linker (residues 90–100).

**Identification of StlSaPI1 homologs**

The search for StlSaPI1 homologs was done using the Blast server. The structural models for the StlSaPI1 homologs were predicted using the AlphaFold server (24) with the option ‘template mode: none’ and the structural alignments were performed with PROMALS3D (25). All homolog model representations were generated with UCSF Chimera (26).

**Quantification and statistical analysis**

All statistical analyses were performed as indicated in the figure legends using GraphPad Prism 6.01 software.

**RESULTS**

**SaPI1 Stl is a tetramer**

To understand the molecular basis of SaPI1 Stl (StlSaPI1) repression, we initiated these studies to solve its atomic structure, either alone or bound to its cognate operator. Although this was not possible, during its purification the chromatographic assays revealed that StlSaPI1 is a tetramer in solution. The StlSaPI1 monomer has 244 residues with a predicted molecular weight (MW) of 29.9 kDa (without the N-terminal His6-tag), but size-exclusion chromatography multi-angle light scattering (SEC-MALS) eluted StlSaPI1 in a single peak with a calculated MW of 117.4 kDa, which corresponds to four molecules of StlSaPI1 (Supplementary Figure S1). This result was interesting because most MGE repressors studied to date form dimers. It raised interesting questions about how StlSaPI1 performs its function and how the SaPI1 inducer, phage 80α Sri protein, promotes SaPI1 induction. Our initial hypothesis was that Sri disrupts the StlSaPI1 oligomeric state, as was observed for StlSaPI monomer (20). However, SEC-MALS analysis of Sri in complex with StlSaPI1 demonstrated that the tetrameric form was not affected by the presence of the inducer (Supplementary Figure S1). Co-expression of Sri and StlSaPI1 formed a complex that eluted later than StlSaPI1 alone and showed a smaller hydrodynamic radius, despite a molecular weight of 128 kDa corresponding to one StlSaPI1 tetramer plus 1 or 2 Sri molecules bound to the tetramer (Supplementary Figure S1). This result suggested that the StlSaPI1 tetramer alone has a structure that is more extended than when it is in a Sri-StlSaPI1 complex, indicating that Sri has the effect of compacting the StlSaPI1 tetramer.

The SEC-MALS results were confirmed by the X-ray crystallographic structure of the Sri-StlSaPI1 complex (Figure 1), which was determined to 2.9 Å resolution by single-wavelength anomalous dispersion (SAD) using the diffraction of a selenomethionine-substituted derivative crystal (Table 1). The asymmetric unit of the crystal contained two StlSaPI1 monomers (subunits A and B) in a dimeric organization around a non-crystallographic two-fold axis, with one Sri molecule binding to the StlSaPI1 subunit A (Figure 1B). The electron density observed in the model suggested that a second Sri molecule binds to the corresponding position of StlSaPI1 subunit B. However, in concordance with our
Figure 1. Structure of the StlSaPI1–Sri complex. (A) Representation of the secondary structure of StlSaPI1 (in pink) and 80α Sri (in green) proteins. The four first α helices of the StlSaPI1 DBDs are coloured in dark pink. (B) Two different views of the 80α Sri-StlSaPI1 complex structure. The molecules in the symmetric unit of the crystal are coloured in dark pink and blue for StlSaPI1 and green for Sri, while the asymmetric molecules are coloured in light colours. The StlSaPI1 DBD surfaces are highlighted in semi-transparent shading. The right part of the figure shows the StlSaPI1 L201 residue located in the helix α8, its sidechain is represented as stick. (C) Structure of the StlSaPI1 subunit A obtained from the Sri-StlSaPI1 complex. The DBD is coloured in green, the helices α5 and α6 are in pink, and the central part of the molecule (β hairpin, α7 and α7–α8 connection) is in orange. The helix α8 is in purple and the C-terminal part (helices α9 and α10) is in blue. (D) Superimposition of the StlSaPI1 DBD in black, the SaPIbov1 Stl DBD (PDB: 6H49) in blue, and the CI lambda phage repressor DBD (PDB: 1LMB) in orange. The four alpha helices are marked.
SEC-MALS results, this additional Sri molecule showed an extremely low occupancy that allowed us to only trace the main chain of fewer than 36% of its residues. In correlation with the low occupancy of the second Sri molecule, two regions of StlSaPI1 subunit B (residues 32–47 and 83–97), which mediate interactions with the inducer in subunit A and connect the DBD and the central part of the protein, present high flexibility that also prevents their tracing. Similarly, we were unable to trace the six N-terminal StlSaPI1 residues which are also placed in this area on subunit A, supporting the idea that the presence of Sri stabilizes the StlSaPI1 N-terminal region. In agreement with our structure and the SEC-MALS experiments, assembly analysis with the PDBfluorescence server (27) indicated that the StlSaPI1 dimer forms a stable tetramer (a dimer of dimers) exploiting the crystallographic two-fold axis. Therefore, the biological assembly of Sri-StlSaPI1 is a box-shaped hetero-octamer of the crystallographic two-fold axis. Therefore, the biological assembly of Sri-StlSaPI1 is a box-shaped hetero-octamer of dimension 95 × 95 × 45 Å, containing two StlSaPI1 homodimers (subunits A–B and A*–B*) and four Sri molecules, two of which were tightly bound (subunits A and A*) and two others with partial occupation (subunits B and B*). In fact, we noticed that some Sri protein eluted alone in a later single peak in our SEC assays. Since Sri can be purified only in a complex with StlSaPI1, the fact that we observed a Sri peak in the chromatographic purification (Supplementary Figure S2) suggested that some Sri molecules were released from the complex because they were weakly bound to StlSaPI1 and/or because the Sri-StlSaPI1 complex has a large dissociation rate constant.

Each StlSaPI1 subunit presents an N-terminal DNA binding domain (DBD) with a conserved HTH-XRE motif formed by 4 α helices (helix α1–α4; residues 1–67) (Figure 1C). This domain is similar in structure to the one in StlSaPI1 (PDB ID: 6H49) (9) and in many phage repressors, including phage λ CI (PDB ID: 1LMB) (28), showing the superimposition of StlSaPI1 DBD with the equivalent Cα atoms of these repressors with a higher root-mean-square deviation (RMSD) of 1.40 and 2.3˚A, respectively (Figure 1D). Conversely, no similar structures were found in the PDB for the rest of the StlSaPI1 repressor. A search for 3D homologs by comparison servers such as DALI or PDBeFold (29) (30), using as prey the structure formed by StlSaPI1 residues 68–244, did not identify any structural homologs to StlSaPI1. This unique architecture is composed of two long α helices (α5 and α6) with a long loop between them that connects the DBDs to the central part of the protein. This central part is formed by two antiparallel strands (β1-β2) in a β hairpin and a short α helix (α7), followed by an extended region without secondary structure (residues 157–191) that projects from one subunit onto the other in the dimer, then returns to the same subunit via a long helix (α8). Finally, two shorter helices (α9 and α10) form the C-terminal part of the protein (Figure 1C). Thus, each subunit is highly elongated with a distance of more than 80 Å between the DBDs and the unstructured region of the protein.

StlSaPI1 self-associates in a unique conformation that has not been previously observed in other repressors, with more than 40% of its residues interacting to form a huge dimerization surface of ∼9230 Å² per subunit (see Supplementary Table S7 for a detailed description of the interaction between the two subunits). In the dimer, 4 helices (α4, α5, α9 and α10) plus the β hairpin of one subunit create a cavity where connecting helices α7* and α8* (residues 154–183) from the other subunit are located, embracing one monomer with the other (Supplementary Figure S3). Although a large number of contacts maintain StlSaPI1 dimers, the α7–α8 connector assembles the most important interactions for dimer formation (Supplementary Table S7). A comparison of StlSaPI1 subunits A and B showed an overall RMSD of 1.5 Å (superimposition of 207 Cα atoms). The differences between both subunits were not uniform along the molecule, being higher towards the N-terminus (RMSD > 2.5 Å) where the HTH domains are located. By contrast, the differences were smaller in the main body of the molecule (RMSD < 1 Å), which is used for oligomerization. These results suggest a high plasticity for the tetramer, which is reflected in the mobility of the DBDs.

Further analysis of the tetrameric state of StlSaPI1 revealed that it presents a reduced oligomerization interface that buries only ∼2150 Å² of the tetramer surface (∼540 Å² per subunit), supporting a dimer-of-dimers organization for the Sri-StlSaPI1 tetramer. The tetramerization surface is generated mainly by the mutual interaction of helices α8 (residues 194–209) from each subunit, forming an anti-parallel four-helix bundle in the tetramer (Figure 1B). These interactions were mainly hydrophobic and provided by the sidechains of residues N193, D194, T197, D200, L201, V204, F205, N208 and K209 that face the centre of the tetramer (Supplementary Table S8).

StlSaPI1 tetramerization is necessary for SaPI1 repression

To gain more insight into the biology of the StlSaPI1 repressor, we analysed whether the tetramer was required for SaPI1 repression. Our previous structural analysis suggested the importance of residue L201 for tetramerization since their mutual interaction projecting from the middle of α8 nucleates the hydrophobic core (Figure 1B, Supplementary Table S8). In support of this, a StlSaPI1 repressor carrying an L201E mutation (StlSaPI1 L201E), which introduces a charged residue into this hydrophobic environment, formed dimers in solution (Supplementary Figure S1) and confirmed the role of this helix in SriStlSaPI1 tetramerization. Furthermore, we solved the structure of the Sri-StlSaPI1 L201E complex, confirming both the dimerization state of the L201E mutant and its ability to interact with Sri, as our previous co-expression and SEC-MALS experiments suggested (Supplementary Figure S1).

The Sri-StlSaPI1 L201E structure was determined at 2.97 Å resolution by molecular replacement using the wt Sri-StlSaPI1 monomer structure that was previously determined as a model. The asymmetric unit of the crystal showed a monomer of SriStlSaPI1 L201E bound to a molecule of Sri (Figure 2). This complex formed a dimer with the symmetric molecules with identical organisation to that observed for the dimer formed by subunits A and B in the crystal asymmetric unit of the wt Sri-StlSaPI1 complex (RMSD of 1.2 Å for the superimposition of 489 Cα atoms of wt dimer with mutant crystallographic dimer), confirming the A–B subunit organization for the dimeric SriStlSaPI1 (Figure 2). StlSaPI1 plasticity was also observed when comparing the subunits
Figure 2. Structure of Sri-StlSaPI1 L201E complex. Structure of the Sri-StlSaPI1 L201E dimer complex obtained by X-ray crystallography. The StlSaPI1 L201E dimer is colored in blue, while the Sri molecules are colored in green and yellow. The sidechain of the mutated E201 residue is represented as stick. The right part of the figure represents an apical view of the Sri-StlSaPI1 L201E structure superimposed with the wt Sri-StlSaPI1 dimer seen in the asymmetric unit of the crystal (subunits A and B, in grey). The RMSD between the wt and the L201E StlSaPI1 dimers is represented.

A and B of the wt StlSaPI1 with those of the StlSaPI1 L201E mutant (RMSDs of 0.9 and 1.6 Å for the superimposition of 243 and 208 Cx atoms of mutant StlSaPI1 with the wt subunits A and B, respectively). The Sri molecule binds to StlSaPI1 L201E in a disposition identical to that previously observed in the wt Sri-StlSaPI1 complex, indicating that the L201E mutation did not affect StlSaPI1 binding to Sri (Figure 2). However, although the Sri fold in both wt and mutant StlSaPI1 complexes is identical (RMSD of 1.5 Å for superimposition of 42 Cx atoms), the 7 C-terminal residues were not visible in the complex with the StlSaPI1 L201E mutant. In the complex with wt StlSaPI1 protein, this portion of Sri was projected into the tetramerization region where it made contact with helix α8. Loss of tetramer organization by the mutation could be detrimental to the stabilization of this region and consequently for complex formation (see below). Corroborating this, the Sri-StlSaPI1 L201E complex was unstable during the purification process and we observed Sri released from the complex during gel filtration chromatography (Supplementary Figure S2).

To test whether the dimeric StlSaPI1 L201E was able to repress SaPI1, we generated a plasmid in which a β-lactamase reporter gene was fused to xis, downstream of str and the Stl-repressed str promoter. This plasmid also encodes the stl in the opposite direction (see scheme in Figure 3A). As a control, we generated a derivative plasmid expressing StlSaPI1 L201E. These plasmids were introduced into strain RN4220 lysogenic for SaPI1 (5). These strains were then treated with mitomycin C (MC) for prophage induction, samples were taken at time zero and after 90 minutes, and the expression of β-lactamase was quantified. In accordance with previous studies (4,5), StlSaPI1 blocked β-lactamase expression in the absence of prophage induction (Figure 3B). Prophage activation expressed the Sri inducer, which derepressed the system promoting the expression of β-lactamase (Figure 3B). In contrast, the plasmid expressing the dimeric StlSaPI1 L201E repressor showed extremely high reporter expression even in the absence of prophage induction (Figure 3B), confirming its inability to cause repression. Not that we tried to generate a SaPI1 derivative encoding the StlSaPI1 L201E mutation, but this was not possible since mutations that negatively affect Stl function are not stable owing to uncontrolled replication of the SaPI (3). In summary, these results demonstrate that the tetrameric structure is required for StlSaPI1 repression.

Characterization of the stl and str promoter regions

Next, we asked why the tetrameric StlSaPI1 is required for SaPI1 repression. To do this, we performed 5´-RACE to identify the stl and str transcription start sites. We also localized the putative RNA polymerase binding sites for both promoters (Figure 3E). While the str promoter showed canonical −10 and −35 sequences, the −35 site of the stl promoter was degenerate (Figure 3E), suggesting that the str promoter was likely stronger than the stl promoter (31). To validate the localization of the putative str promoter, we made use of the reporter plasmid in which the blaZ reporter gene was fused to xis (see scheme in Figure 3A), but now with a single nucleotide mutation in the −35 site of the str gene. This plasmid was introduced into the strain lysogenic for 80α and the expression of β-lactamase was measured. We found that the single nucleotide mutation eliminated transcription of the str promoter after prophage induction (Figure 3C).

To characterize the stl promoter, we made a transcriptional fusion of the stl promoter to the blaZ reporter gene (see Figure 3E). We also generated a derivative with a single point mutation in the −35 site of the stl promoter.
Figure 3. The StlSaPI1 tetramer is required for SaPI1 repression. (A) Schematic representation of the pCN41 derivative reporter plasmid used to analyse StlSaPI1 repression. In the absence of prophage induction, the expression of SaPI1 and blaZ genes is repressed by StlSaPI1. Induction of the 80a prophage results in expression of the SaPI1 inducer Sri, which promotes the expression of the SaPI1 genes, including the blaZ reporter. (B) Lysogenic strains for phage 80a, carrying pCN41 derivative plasmids expressing either the wt StlSaPI1 or the mutant StlSaPI1 L201E, were MC-induced (IN) or not (UN), and the expression of the blaZ reporter analysed at time zero (t = 0) or 90 min (t = 90) after MC induction. The means and standard deviation from three independent experiments are represented. A two-way ANOVA comparison was performed to compare the different samples mean between StlSaPI1 and StlSaPI1 L201E (**** P < 0.0001; ns, P = 0.1234). (C) Characterization of the str promoter. The strains lysogenic for phage 80a, carrying pCN41 derivative plasmid containing either the wt str promoter (Pstr), or one carrying a point mutation in the putative –35 site of the Pstr (mutant Pstr), were MC-induced (IN) or not (UN) and the expression of the blaZ reporter was analysed 90 min after prophage induction. The means and standard deviation from three independent experiments are represented. A two-way ANOVA comparison was performed to compare the IN wt Pstr mean with the IN Pstr mutant mean (****, P < 0.0001). (D) Characterization of the stl promoter. RN4220 strains carrying pCN41 derivative plasmids containing either the wt stl promoter (Pstl) or one carrying a point mutation in the putative –35 site of the Pstl (mutant Pstl), were analyzed at zero min. The means and standard deviation from three independent experiments are represented. A one-way ANOVA comparison was performed to compare the wt Pstl mean with the Pstl mutant mean (****P < 0.0001). (E) DNA sequence of the SaPI1 stl-str intergenic region. The transcription start sites are represented with arrows and the –10 and –35 sequences are highlighted in blue for the Pstr, or in green for the Pstl. The stl promoter region that was cloned into the pCN41 plasmid for the characterization of the Pstl is marked with a bracket.

These plasmids were then introduced into the non-lysogenic RN4220 strain and the expression of reporter was measured. While the wt plasmid showed high β-lactamase expression, the plasmid carrying the point mutation did not (Figure 3D). Taken together, point mutations in the –35 sites of the str and stl promoters completely abolished their transcription, confirming the identity of both promoters.

The SaPI1 stl-str intergenic region contains 8 StlSaPI1 binding sites

We next focused on the SaPI operators to determine if their organization reflected the four DBDs in a StlSaPI1 tetramer. Foot-printing experiments using StlSaPI1 with the SaPI1 stl-str intergenic region revealed two protected regions, separated by 24 bp, in the top and bottom strands (Figure 4A). We used this intergenic region because previous studies have shown that SaPI Stl proteins bind to it (5,9), and our reporter assays showed that it is regulated by Stl. Detailed analysis of the protected regions identified eight putative StlSaPI1 binding sites organized as four distinct operators (1–4) (Figure 4B). Operators 3 and 4 appear to represent a higher affinity site because they were fully protected at lower concentrations of StlSaPI1 and required a higher concentration of Sri to lose protection compared with the region containing operators 1 and 2. Each of the putative operators shows almost perfect palindromic organization, containing two inverted 6 bp repeats with the consensus sequence TG-TACT (called boxes A and B) separated by 3 bp (Figure 4B). Importantly, operators 1 and 2 overlap with the -35 sites of
Figure 4. Identification of the StlSaPI1 binding sites in the SaPI1 stl-str intergenic region. (A) Foot-printing experiments carried out with the SaPI1 stl-str intergenic region and the StlSaPI1 protein, alone or in the presence of Sri. Protein concentrations used in the experiment are represented in the figure. The protected regions associated with operators 1 and 2 (1+2) or 3 and 4 (3+4) are shown. Regions 1+2 and 3+4 correspond to regions with low and high affinity for StlSaPI1, respectively. (B) Schematic representation of the four operator sites for StlSaPI1. Both palindromic boxes of each operator are highlighted as A and B. The Pstr and Pstl transcription start sites are represented by blue or green arrows, respectively. The –10 and –35 sequences from both promoters are also highlighted in blue and green, respectively.

Since classical repressors containing HTH-XRE domains are usually dimers that bind to palindromic operators, the existence of four operators suggested that two StlSaPI1 tetramers bind to this region (corresponding to four classical dimers). To test this, we first confirmed that the 4 identified operators are recognized and bound by the StlSaPI1 DBDs. To simplify the interpretation of our results, we initially performed electrophoretic mobility shift assays (EMSA) using the StlSaPI1 L201E mutant since the altered residue does not affect the conformation of the DBD domain or its ability to bind operators. We hypothesized that each of the DBDs in the StlSaPI1 L201E mutant would bind as a dimer to each of the four operators, independently. To analyse the binding of StlSaPI1 L201E, we generated a set of DNA probes that each contained one of the operators. As shown in Figure 5A, StlSaPI1 L201E bound all 4 DNA probes, showing the highest affinity for operators 3 and 4 and corroborating the foot-printing results.

Next, protein–DNA binding kinetics assays were performed by biolayer interferometry to obtain the affinity constant ($K_D$) of both the wt StlSaPI1 and the L201E mu-
Figure 5. Characterization of the StlSaPI1 binding sites present in the stl-str intergenic region. (A) EMSAs performed using the StlSaPI1 L201E mutant protein (0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 μM per well), and DNA probes (1 μM) containing only one operator site per probe. The free probe band is marked with a black arrow, while the Stl-DNA complex bands are highlighted with a red arrow. (B) Biolayer interferometry assays with both the StlSaPI1 and the StlSaPI1 L201E proteins and each of the probes containing one of the four individual operators in the stl-str intergenic region. A negative probe, operator 3 with the Stl binding sites mutated to Adenines, was used as nonspecific DNA binding control. ‘K<sub>D</sub>’ affinity constant, ‘k<sub>on</sub>’ association constant, ‘k<sub>off</sub>’ dissociation constant, ‘nd’ non signal detected. (C) As for (A) but using StlSaPI1 L201E mutant protein (1, 2, 3, 4, 5, 6, 7 and 8 μM per well) and a DNA probe (1 μM) containing the full stl-str intergenic region with the four operators. (D) As for (C) but using the wt StlSaPI1 protein. (E) As for (D) but using a negative probe with the four operators with the consensus regions mutated to Adenines.

The results showed that StlSaPI1 bound to each of the operators with K<sub>D</sub> values ranging from 0.7 to 10 nM (Figure 5B), while StlSaPI1 L201E showed similar K<sub>D</sub> values of 17–30 nM. Of note, the constants for operators 1, 2 and 3 were 1 to 2 orders of magnitude lower than those observed for the tetrameric wt StlSaPI1 repressor (Figure 5B). No binding was observed with the mutated operator 3, confirming DNA recognition specificity. While the binding kinetics for the StlSaPI1 L201E mutant fit a 1:1 model (operator:dimer), we observed more complex sensograms with wt StlSaPI1 which better corresponded to a mass transport model (Supplementary Figure S4). These observations also explain the differences in the K<sub>D</sub> values observed between the wt and the mutant StlSaPI1, since with the wt protein we visualized an avidity effect owing to the tetramer that simultaneously bound two independent and immobilized operators (one per dimer). These results also indicated that StlSaPI1 and StlSaPI1 L201E bound operators through the dimer formed by subunits A and B.
Two Stl tetramers bind to the stl-str intergenic region

Having demonstrated the existence of four operators in the stl-str intergenic region, we then analysed the interaction of the wt StlSaPI1 and the StlSaPI1 L201E mutant with a DNA probe containing all four operators. Here, four different protein–DNA species were observed in the presence of the StlSaPI1 L201E mutant protein (Figure 5C); interestingly, only two different protein–DNA species were obtained with the wt StlSaPI1 (Figure 5D). As a control, a probe with all of the consensus sequences mutated to adenine was used to confirm DNA-binding specificity of the different proteins (Figure 5E). SEC-MALS characterization of the sample corresponding to the DNA probe in the presence of an excess (4-fold molar ratio) of wt StlSaPI1 showed two peaks. The first peak had a calculated MW in close agreement to that of a complex of two tetramers with one DNA probe, while the second peak had a MW almost identical to that of a StlSaPI1 tetramer (Supplementary Figure S5) that was likely attributable to the excess protein. Both the EMSA and SEC-MALS results supported the hypothesis that StlSaPI1 tetramers are bivalent and bind two DNA operators. Therefore, two tetramers would be enough to occupy the four operators in stl-str intergenic region.

Our previous results showed that StlSaPI1 L201E was unable to repress SaPI1, even though it binds to the 4 Stl boxes with high affinity. This result implied that the StlSaPI1-DNA interaction was more complex than just DNA binding. The existence of 4 operators with different affinities for StlSaPI1, together with the requirement for 2 StlSaPI1 tetramers with high flexibility in their DBD domains, prompted us to propose two different models for SaPI1 repression (currently under investigation) (Figure 6). In the first model, which is supported by the foot-printing experiments, one tetramer binds with high affinity to operator sites 3 and 4, while the other tetramer binds to sites 1 and 2. In order to bind both sites at the same time, StlSaPI1 must recognize an inverted palindrome of each operator that would generate two canonical operators (3A-4B and 1A-2B). In this case, the binding sites are separated by 22–27 bp instead of 3 bp (Figure 6B). The length of this spacer would imply that the DNA-binding helices must be more than 60 Å apart, which should not be a problem for StlSaPI1 since the Sri-StlSaPI1 structure shows that the α3 helices are separated by ∼61 Å in the dimer. Although this type of separation between palindromes is unusual, it has recently been observed that the transcriptional activator AimR from B. subtilis phage SPbeta recognizes an operator with similar organization, where highly flexible DBDs more than 75 Å apart bridge a 25 bp spacer (32,33). A protein-protein interaction between both tetramers could create a bigger protein–DNA complex to stabilize Stl repression, which could explain why StlSaPI1 tetramers are required for SaPI1 repression. Another possibility is that one dimeric part of the first tetramer initially binds with high affinity to operator 3, and then the binding of the second dimeric part of this tetramer to operator 2 induces a DNA torsion that is facilitated by the high A/T content of the inter-operator spacer (Supplementary Figure S6). Next, a second tetramer stabilizes the protein–DNA complex via bivalent binding to operators 1 and 4 (Figure 6C, Supplementary Figure S6). An alternative to the sequential entry of tetramers in this model, two tetramers could bind simultaneously to operators 3 and 4 by one of their dimeric parts, inducing DNA torsion by the binding of their second dimeric parts to operators 2 and 1, respectively. Such multiple interactions would be possible because of the high flexibility observed in the StlSaPI1 DBDs (see below), and because of the ability of these domains to bind, bend and twist DNA. We propose that either of these models could stabilize the complex, preventing RNA polymerase from binding to the stl and str intergenic region - a process that is also supported by the observation that StlSaPI1 binds to the –35 sites of the stl and str promoter (located in operators 1 and 2, respectively; Figure 4B). Importantly, in the case of the stl promoter, this repression would reduce StlSaPI1 formation. Therefore, both models propose that the second tetramer controls the amount of StlSaPI1 that will be produced, which is crucial for the control of the system. When StlSaPI1 is in excess, binding of the second tetramer to operator 1 will reduce stl expression; when StlSaPI1 decreases, the absence of the second tetramer will allow stl expression. Although we have not yet confirmed either model, binding of StlSaPI1 L201E to individual operators and the EMSA results observed for wt and mutant repressors support the latter model, while the foot-printing data are more consistent with the former. Further studies are required to resolve this mechanism.

Molecular basis of SaPI1 de-repression by Sri

Our results demonstrate that in each operator, a dimer with two StlSaPI1 DBDs binds to an operator with two palindromic sequences separated by 3 bp. However, in the X-ray crystallography structure of the Sri-StlSaPI1 complex, the distance observed between the two DBDs (> 60 Å) was greater than that required to bind to the Stl boxes in the operators (30 Å, Supplementary Figure S7). Sri is a 6.2 kDa protein (52 residues) composed of a 3-helix bundle (α1–α3) followed by an extended non-structured C-terminal tail (residues 43–51) (Figure 1). A DALI search showed that Sri is structurally similar (1.28 Å RMSD over 41 Cα superimposed) to the phage 77 ORF104, whose structure was previously solved in complex with DnaI, its cellular partner (34) (PDB ID 5HE9). When complexed with StlSaPI1, Sri is inserted in the StlSaPI1 tetramer, interacting with 3 of the 4 StlSaPI1 subunits and burying ∼1500 Å² of its surface, which corresponds to ∼33% of the Sri molecular surface. Each Sri molecule mainly interacts with the StlSaPI1 DBD of one subunit by using helices α2 and α3 to contact StlSaPI1 helices α1, α4 and α5 (Supplementary Table S9). In addition, the Sri α3 helix interacts with the C-terminal α10 helix of the symmetrically related subunit (A*) in the StlSaPI1 tetramer; and its extended C-terminal tail is positioned over two α8 helices (subunits A* and B), which nucleates StlSaPI1 tetramerization by interactions with subunit B (Supplementary Table S9). By performing this network of interactions, Sri fixes the StlSaPI1 DBDs to the main body of the StlSaPI1 tetramer, restricting its conformational freedom and compacting the StlSaPI1 tetramer structure, as our SEC-MALS experiments corroborate (Supplementary Figure S1). Low occupancy of Sri in the second binding site of the StlSaPI1 dimer correlates
Figure 6. Proposed models for StlSaPI1 repression. (A) Representation of the StlSaPI1 tetramer and the SaPI1 stl-str intergenic region. The two dimers that from the StlSaPI1 tetramer are dark and light grey and the mobility of the DBDs is represented by dashed lines. The 4 operators are represented with different colors and the inverted repeats are named A and B. (B) Model 1. When StlSaPI1 concentration is low, one tetramer binds the high-affinity operators 3 and 4, with the DBDs from the dimer binding to 3A and 4B to repress str expression. When StlSaPI1 concentration increases, a second tetramer binds to 1A and 2B to repress stl expression. Since tetramer formation is required for SaPI1 repression, the two tetramers interact somehow to stabilize the complex. (C) Model 2. At low StlSaPI1 concentration, one dimer of the StlSaPI1 tetramer binds to both repeats (A and B) from operator 3, while the other binds to the repeats present in operator 2, creating a torsion in the DNA which favors str repression. When StlSaPI1 concentration increases, a second tetramer binds in a similar manner to operators 1 and 4, stabilizing the complex and increasing repression of the system, represented in black arrows. In grey arrows an alternative model is represented with a sequential entry of the tetramers, two tetramers could bind simultaneously to operator 3 and 4 by one of their dimeric parts, inducing DNA torsion by the binding of their second dimeric part to operators 2 and 1, respectively.

with the high flexibility of the DBD and supports Sri function. Therefore, we hypothesize that Sri de-represses SaPI1 by fixing the StlSaPI1 DBDs (Figure 1, Supplementary Table S9) in a conformation that is not compatible with the binding of the Sri-StlSaPI1 complex to the StlSaPI1 operators in the stl-str intergenic region (Supplementary Figure S7).

To test this hypothesis, we first validated our structural data for the StlSaPI1-Sri complex. To do this, we mutated the StlSaPI1 residue Y76 to Alanine (StlSaPI1 Y76A), which we deduced was important for the stabilization of the StlSaPI1-Sri complex by projecting its side chain into a hydrophobic pocket generated by StlSaPI1 residues W14, M63, F69, I72 and Y76 (Figure 7A, Supplementary Table S8). Pull-down assays confirmed that the StlSaPI1 Y76A repressor was unable to bind to Sri (Figure 7B). To show that this mutation affected only the interaction with Sri, but not the ability of StlSaPI1 Y76A to repress the island, we again used the β-lactamase reporter plasmid (see scheme in Figure 3A) with either the wt or the StlSaPI1 Y76A mutant repressor. These plasmids were introduced into the 80α lysogen and expression from the Stl-repressed str promoter was measured after induction of the 80α prophage. Compared with
Figure 7. Characterization of the StlSaPI1-Sri interaction. (A) Details of the StlSaPI1 (in pink) and Sri (in green) interaction. The StlSaPI1 Y76, S13, M16 and K20 Sri residue sidechains are represented as sticks. (B) SDS-PAGE gel after pull-down experiments in which the His6 tagged Sri protein was co-expressed either with the wt StlSaPI1 or the StlSaPI1 Y76A proteins. Uninduced (UN), induced (IN) and eluted (E) from the Ni2+ column. Note the absence of the prey band due to the loss of solubility of Sri in the absence of StlSaPI1. (C) Strains lysogenic for phage 80a, carrying pCN41 derivative plasmids expressing either wt StlSaPI1 or StlSaPI1 Y76A, were MC-induced (IN) or not induced (UN) and expression of the bluZ reporter analysed 90 min after prophage induction. The means and standard deviation from three independent experiments are represented. A t-test comparison was performed to compare IN StlSaPI1 Y76A mean with IN StlSaPI1 mean (****P < 0.0001). (D) Lysogenic strains for phage 80a, carrying wt SaPI1 tst::tetM or a derivative SaPI1 tst::tetM carrying the StlSaPI1 Y76A mutation, were MC-induced (IN) or not induced (UN), and the transfer of the island quantified. The means and standard deviation from three independent experiments are represented. A t-test comparison was performed to compare each the StlSaPI1 Y76A mean with the StlSaPI1 mean (****P < 0.0001).

that observed for wt StlSaPI1, no significant activity was observed in the plasmid expressing StlSaPI1 Y76A under all of the conditions tested, confirming that the mutant protein was still able to repress the island but was insensitive to the Sri inducer (Figure 7C). Finally, we tested the impact of the StlSaPI1 Y76A mutation in vivo. We generated a SaPI1 tst::tetM derivative island expressing StlSaPI1 Y76A. Note that this island carries an antibiotic resistance marker which facilitates transfer studies. The strain was then lysogenized with phage 80a, and the transfer of the island was analysed after induction. As a control, we included a strain lysogenic for 80a carrying the wt SaPI1 tst::tetM. Transfer of the SaPI1 mutant was significantly reduced compared to the wt SaPI1 (Figure 7D). Taken together, these results validate the Sri-StlSaPI1 interactions revealed by the X-ray crystallographic data.

Though we were unable to obtain the structure of StlSaPI1 alone or complexed with its cognate DNA, we obtained low resolution structural information about this protein in solution using small-angle X-ray scattering (SAXS). First, we generated a SAXS data set for the Sri-StlSaPI1 complex and for StlSaPI1 alone merging 14 and 49 frames of SAXS data for which a constant $R_g$ of 39.7 and 41.1 Å was estimated, respectively. The maximum particle dimension $D_{\text{max}}$ was 127.3 and 139.1 Å for the Sri-StlSaPI1 complex and for StlSaPI1 alone, respectively (Supplementary Figure S8). The mass of a Sri-StlSaPI1 monomer (including a His6 tag on Sri) is 38 997 Da, and the mass of StlSaPI1 is 29 398 Da, which translates to masses of 155 988 Da for a tetramer of Sri-StlSaPI1 or 117 592 Da for a tetramer of StlSaPI1. Molecular weight analysis by Bayesian inference (22) in Primus estimated $M = 124 450$ Da (46.10% probability) with a credibility interval of [111 250, 134 300] (99.40% probability) for StlSaPI1-Sri and $M = 101 050$ Da (32.44% probability) with a credibility interval of [92 650, 111 250] (95.59% probability) for StlSaPI1 alone. The SAXS analysis was therefore
strongly suggestive of a tetramer for both Sri-StlSaPI1 and StlSaPI1 alone. A model for a StlSaPI1 tetramer was generated by removing the coordinates of Sri from the X-ray crystallography structure of the StlSaPI1-Sri complex (Figure 8A). US-SOMO (35) was used to compute $R_c = 36.8$ A for this model, indicating that the model extracted from the crystal structure is more compact than the StlSaPI1 tetramer in solution. Thus, the mobility of the StlSaPI1 DNA-binding domains (DBDs) was modelled using EOM (23) (Figure 8B, Supplementary Figure S8). 10 000 models were generated in which the C-terminal domains (CTD, residues 101–247) were kept in the conformation observed by X-ray crystallography but the DBDs (residues 1–89) were allowed to adopt positions consistent with their connection to the CTD via a native-like flexible linker (residues 90–100). In support of this, the crystallographic structure of Sri-StlSaPI1 showed high mobility in the StlSaPI1 DBDs when Sri was weakly bound (subunits B and B*, Figure 1B), as well as differences between subunits in the dimer (Figure 1B), supporting the notion that the structure of these DBDs is highly plastic in the absence of Sri and is compatible with binding to operators. The disposition of the StlSaPI1 DBD was more extended compared with the DBD of the complex Sri-StlSaPI1, which was also shown in our SEC-MALS results where a higher hydrodynamic volume was observed for StlSaPI1 than for the Sri-StlSaPI1 complex (Supplementary Figure S1).

These results indicate that SaPI1 de-repression involves a mechanism different from that used by SaPI1bmr. While the latter involves separation of the StlSaPI1bmr dimer by the inducing dUTPases (9), our data show that Sri de-represses SaPI1 by inducing a conformational change in the StlSaPI1 DBDs, preventing the binding of these domains to their operators.

StlSaPI1 homologs are widespread in nature

Because of the unusual nature of the SaPI1 repression system, we wanted to know if it was exclusive to this island or more widespread in nature. In a search for StlSaPI1-like homologs in the publicly accessible databases, different homologs were found in Staphylococci and different species of Bacillus and Virgibacillus, which have a sequence identity (compared to StlSaPI1) that ranges from 26 to 35% (Supplementary Table S10). Importantly, while the StlSaPI1-like homologs in Staphylococcus spp. were encoded by different members of the PICI family (Supplementary Table S10), suggesting a mechanism of induction in common with that reported here for SaPI1, the homologs present in the other genera were encoded by MGEs other than PICIs. Moreover, the 3D models obtained by the AlphaFold server for these proteins confirmed their structural homology with StlSaPI1 (TM-scores 0.45–0.6; Figure 9A and Supplementary Figure S9), and the detailed analysis of the secondary structure of these homologs showed that the DBD (present in the first 3 α helices) and the dimerization (region between helices a7 and a8) or tetramerization (helix a8) key residues were extremely well conserved among these proteins (Figure 9B). These results confirm the discovery of a new family of repressors involved in gene transfer and bacterial evolution.

DISCUSSION

Many MGEs, including prophages, PICIs or ICEs, control their life cycles by expressing master repressors that maintain these elements integrated into the bacterial chromosome. Importantly, the expression of these repressors must be precisely controlled since an excess of the repressor would impede the induction and transfer of the element, while a reduced expression would generate either the loss or the activation of the element under unfavourable conditions.

To ensure tightly-regulated control, SaPI1 has evolved a unique system involving two StlSaPI1 tetramers and four operators. As previously mentioned, we proposed here two different models for SaPI1 repression (Figure 6). The best characterized repressor so far is CI from phage λ. CI represses both cl and cro expression by binding at operators $\text{OL}$ and $\text{OR}$, each composed of three repressor binding sites named $\text{OL1}$, $\text{OL2}$ and $\text{OL3}$, or $\text{OR1}$, $\text{OR2}$ and $\text{OR3}$, respectively (36). Two CI dimers bind tightly and cooperatively to $\text{OL1}$ and $\text{OL2}$, creating a tetramer that represses expression from the $\text{pL}$ promoter (37). A similar tetramer bound structure is formed after the binding of two CI dimers to $\text{OR1}$ and $\text{OR2}$, repressing in this case the expression from the $\text{pR}$ promoter (37). To generate a more stable repression system, these two tetramers interact to form an octamer looping the DNA between the $\text{OL}$ and $\text{OR}$ operator regions (11,38). While StlSaPI1 and CI repression involve the formation of tetramers, these two systems are completely different, both structurally and mechanistically, probably in response to the different ways that phages and SaPIs are induced. In λ, the two tetramers and the octamer appear only after the binding of the CI dimers to their cognate binding sites, while StlSaPI1 is always a tetramer. Moreover, our results indicate that the binding of the two StlSaPI1 tetramers to their cognate DNAAs is not cooperative but sequential. Structurally, these two repressors are completely unrelated, except for their DBD regions. Functionally, they also work in completely different ways. Thus, after activation of the bacterial SOS response, the RecA* protein will promote the autocleavage of CI, disrupting dimer, tetramer and octamer formation, while in the case of SaPI1, the Sri protein does not affect tetramerization of StlSaPI1 but will force the StlSaPI1 DBDs to adopt a conformation that prevents their interaction with these cognate DNA boxes.

Two other systems involving tetramer formation have been described in the control of the transfer of different MGEs. The repressor (Rep) of temperate Salmonella phage SPC32H can reversibly assemble into two oligomeric states (dimer and tetramer) in a concentration dependent manner (39). As with StlSaPI1, Rep binds to DNA as a tetramer, though these tetramers are structurally different. Contrary to what we observed with the StlSaPI1 $\text{L201E}$ dimer, the dimeric Rep protein binds DNA weakly even at high concentrations. This difference can be easily explained since it has been proposed that the dimer pairs required for binding to the palindromic DNA sites originate from different dimers in the tetrameric Rep (39), while in the StlSaPI1 they come from the same dimer. Another difference between these systems relates to how they are de-repressed. While the SaPI1 inducer Sri is a monomer that forces a confor-
Figure 8. The StlSaPI1 DBDs are flexible in solution. (A) StlSaPI1 tetramer structure was generated by removing the Sri coordinates from the X-ray crystallography structure of the StlSaPI1-Sri complex. Residues 90–244 are in grey and DBD surfaces are in red. (B) Model with the DBD conformers which best fit to the SAXS data via the ensemble optimization method for StlSaPI1 in solution. StlSaPI1 residues 90–244 are in grey and the DBD conformers are represented in surface with different transparencies proportional to the percentage of that model in the total ensemble. The fit was obtained with ~50% of model 1 (DBDs in orange), 25% of model 2 (DBDs in green) and 13% of models 3 (DBDs in blue) and 4 (DBDs in purple).

nutional change in the StlSaPI1 DBDs, the SPC32H anti-repressor Ant is a tetramer that binds to two dimeric Reps, breaking tetramer formation. Another important difference between the two systems is that only one tetramer is required for phage SPC32H repression (39), but two are required for SaPI1 repression.

The second system is from the Enterococcus faecalis conjugative plasmid pCF10. This plasmid encodes PrgX, which is a repressor that blocks the expression of genes involved in the conjugative transfer of this plasmid. As with StlSaPI1, PrgX is a tetramer that binds to two different operator regions and forces a looping of the DNA (40). However, Rep, PrgX and StlSaPI1 are unrelated in structure. Moreover, the mechanism involving pCF10 transfer is also different from that observed for SaPI1. The transfer of the pCF10 plasmid occurs in response to an intracellular pheromone signal, a peptide called cCF10 with the sequence LVTLVFV. As with Rep, binding of the cCF10 inducer to PrgX destabilizes the PrgX tetramer and promotes conjugation (40).

The new repression mechanism described here for SaPI1 is possible due to the localization of the StlSaPI1 DBDs in the tetramer. Canonical members of the HTH-XRE family of repressors dimerize through their α5 helix. In StlSaPI1, the α5 helix connects the DBDs with the rest of the protein by a long loop which confers high mobility for DNA recognition and binding (Figure 1C). The flexibility of the StlSaPI1 DBDs was obvious when we compared the four DBD domains in the Sri-StlSaPI1 X-ray crystallographic structure versus the SAXS data for StlSaPI1. By interacting with StlSaPI1 DBD helices α1, α4 and α5 and two other subunits in the tetramer, Sri maintains the StlSaPI1 DBDs fixed in a conformation that prevents their binding to operators. However, in the absence of Sri, the DBDs showed a more extended localization that allow them to interact with their cognate DNA boxes (Supplementary Figure S7). This plasticity in StlSaPI1 provides conformational freedom to the DBDs and is likely to be the origin of our inability to obtain X-ray structure crystallographic structures for StlSaPI1 and StlSaPI1 L201E.

Another interesting feature of the regulatory system is that the primary role of the phage-encoded SaPI1 inducer is not to induce the island but to interact with the cellular DnaI protein (34), slowing down bacterial replication and facilitating phage reproduction. Thus, SaPI1 has evolved a repressor that uses a conserved phage protein as its inducer. Since DnaI and StlSaPI1 are completely unrelated in sequence and structure, how this small protein interacts with two unrelated proteins to perform two different func-
Figure 9. StlSaPI1 repressor conservation in other species. (A) StlSaPI1-like repressors present in other bacterial species were modelled using Alphafold server. The StlSaPI1 monomer structure in the left part of panel A coloured as follows: the DBD is coloured in green, the helices $\alpha_5$ and $\alpha_6$ are in pink, the central part of the molecule ($/H_9252$ hairpin, $/H_9251$ $/H_9251$-$/H_9251$ connection) is in orange. The helix $/H_9251$ $/H_9251$ is in purple and the C-terminal part (helices $/H_9251$ $/H_9251$) is in blue. The StlSaPI1-like repressor models are represented and coloured based on their residue conservation: blue for non-conserved (0), red for conserved (1) and white for similar residues (0.5). The TM-scores and RMSD for the models compared with the StlSaPI1 structure are indicated in italics in brackets.

(B) Structural alignment of StlSaPI1 and StlSaPI1-like repressors. The residues forming the $/H_9251$ helices or $/H_9252$ strands are coloured in red and blue, respectively. The residue conservation amongst the different proteins is represented on the alignment (with 9 indicating conservation, and 5 similarity). The StlSaPI1 secondary structure is represented below the alignment with colours defined in panel A.

Figure 9. StlSaPI1 repressor conservation in other species. (A) StlSaPI1-like repressors present in other bacterial species were modelled using Alphafold server. The StlSaPI1 monomer structure in the left part of panel A coloured as follows: the DBD is coloured in green, the helices $\alpha_5$ and $\alpha_6$ are in pink, the central part of the molecule ($/H_9252$ hairpin, $/H_9251$ $/H_9251$-$/H_9251$ connection) is in orange. The helix $/H_9251$ $/H_9251$ is in purple and the C-terminal part (helices $/H_9251$ $/H_9251$) is in blue. The StlSaPI1-like repressor models are represented and coloured based on their residue conservation: blue for non-conserved (0), red for conserved (1) and white for similar residues (0.5). The TM-scores and RMSD for the models compared with the StlSaPI1 structure are indicated in italics in brackets.

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Data Availability
Atomic coordinates and structure factors have been deposited at the RCSB Protein Data Bank (PDB) (PDB code 7P4A for StlSaPI1 and 1ZVI for StlSaPI1 L201E).

Supplementary Data
Supplementary Data are available at NAR Online.
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