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Moonlighting bacteriophage proteins derepress staphylococcal pathogenicity islands

María Ángeles Tormo-Más¹, Ignacio Mir¹, Archana Shrestha², Sandra M. Tallent², Susana Campoy³, Íñigo Lasa⁴, Jordi Barbé³, Richard P. Novick⁵, Gail E. Christie², and José R. Penadés^{1,6}

¹Centro de Investigación y Tecnología Animal, Instituto Valenciano de Investigaciones Agrarias (CITA-IVIA), Apdo. 187, Segorbe, Castellón 12400, Spain.

²Department of Microbiology and Immunology, Virginia Commonwealth University School of Medicine, Richmond, Virginia 23298-0678, USA.

³Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Barcelona 08193, Spain.

⁴Instituto de Agrobiotecnología, CSIC-Universidad Pública de Navarra, Pamplona, Navarra 31006, Spain.

⁵Skirball Institute Program in Molecular Pathogenesis and Departments of Microbiology and Medicine, New York University Medical Center, 540 First Avenue, New York, New York 10016, USA.

⁶Departamento de Química, Bioquímica y Biología Molecular, Universidad Cardenal Herrera-CEU, Moncada, Valencia 46113, Spain.

Abstract

Staphylococcal superantigen-carrying pathogenicity islands (SaPIs) are discrete, chromosomally integrated units of ~15 kilobases that are induced by helper phages to excise and replicate. SaPI DNA is then efficiently encapsidated in phage-like infectious particles, leading to extremely high frequencies of intra- as well as intergeneric transfer^{1–3}. In the absence of helper phage lytic growth, the island is maintained in a quiescent prophage-like state by a global repressor, StI, which controls expression of most of the SaPI genes⁴. Here we show that SaPI derepression is effected by a specific, non-essential phage protein that binds to StI, disrupting the StI–DNA complex and thereby initiating the excision-replication-packaging cycle of the island. Because SaPIs require phage proteins to be packaged^{5,6}, this strategy assures that SaPIs will be transferred once induced. Several different SaPIs are induced by helper phage 80α and, in each case, the SaPI commandeers a different non-essential phage protein for its derepression. The highly specific

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Correspondence and requests for materials should be addressed to J.R.P. (penades_jos@gva.es).

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interactions between different SaPI repressors and helper-phage-encoded antirepressors represent a remarkable evolutionary adaptation involved in pathogenicity island mobilization.

Pathogenicity islands have a major role in spreading virulence genes among bacterial populations. A notable example are the phage-related pathogenicity islands of staphylococci, the SaPIs, which are responsible for the inter- as well as intrageneric spread of toxins—such as TSST-1 (toxic shock syndrome toxin) and other superantigens—through the exploitation of specific staphylococcal helper phages for high-frequency transfer within phage-encoded particles⁷. Stable maintenance of SaPIs in the absence of helper phage requires a SaPI-encoded repressor, *Stl*, which, like classic prophage repressors, binds to a region between two divergent promoters that initiate the major SaPI transcripts and thus inhibits expression of most of the SaPI genes⁴. Mutations inactivating *stl* cause SaPI excision and replication in the absence of a helper phage⁴, suggesting that the primary regulatory function of the helper phage is to relieve *Stl* repression. To elucidate phage-mediated SaPI induction, we exploited the documented interference of SaPIs with the growth of their helper phages, which reduces phage burst size by 10–100 fold¹ and blocks plaque formation. We reasoned that phage mutants unable to relieve *Stl* repression would form plaques on a SaPI-containing strain because SaPI genes interfering with phage lytic growth would not be expressed. We used two different, well studied helper phages, 80 α (GenBank accession NC_009526) and ϕ 11 (NC_004615), and three different SaPIs—SaPI1, SaPIbov1 and SaPIbov2⁷. All SaPIs encode an *Stl* homologue, but these proteins are very poorly conserved (Supplementary Fig. 1). Whereas ϕ 11 induces only SaPIbov1, 80 α has been shown to induce at least five different SaPIs (SaPI1, SaPI2, SaPIbov1, SaPIbov2 and SaPIin1⁷) with widely divergent *Stl* proteins, raising the question of how the putative 80 α derepressor protein could have such broad specificity.

Spontaneous 80 α mutants able to form plaques on *Staphylococcus aureus* strain RN4220 containing SaPIbov1 were readily obtained. These mutant phages had lost the ability to mobilize the island, consistent with failure to relieve *Stl*-mediated repression. Eighteen independent SaPIbov1-resistant 80 α mutants carried point mutations in open reading frame (ORF)32, a gene annotated as *dut* based on homology with dUTPases (Supplementary Fig. 2). Missense and nonsense mutations were obtained, suggesting that this gene was both necessary for SaPIbov1 induction and non-essential for the phage. This was confirmed by the introduction of an inframe deletion in 80 α *dut*, which also eliminated SaPIbov1 mobilization but did not impair phage growth (Table 1). Similar results (Table 1 and Fig. 1a) were obtained by deleting the *dut* homologue of ϕ 11, another phage that can mobilize SaPIbov1, confirming the role of *dut* in SaPIbov1 induction.

As mentioned earlier, 80 α mobilizes a number of different SaPIs. Interestingly, the 80 α SaPIbov-resistant *dut* mutants, which plate normally on a SaPIbov1-containing strain, were still unable to form plaques on strains containing either SaPI1 or SaPIbov2, and were undiminished in mobilization of either of these islands (Table 1). This observation raised a surprising possibility for helper-phage–SaPI specificity; namely, that phage 80 α possesses further genes for derepressing these other SaPIs. Using the same selection strategy, 80 α mutants resistant to SaPI1 and SaPIbov2 were isolated. SaPI1-resistant mutations were found in ORF22 (hereafter called *sri*), which encodes a DnaI binding protein that inhibits staphylococcal replication⁸. SaPIbov2-resistant mutations were found in ORF15, which encodes a small protein of unknown function. Construction of inframe deletions confirmed that these two extra phage genes were also non-essential and, once again, specific for inducing the SaPI on which they were selected (Table 1 and S.M.T., P. K. Damle, A.S. and G.E.C., unpublished data). Phage ϕ 11, which cannot induce SaPI1 or SaPIbov2, lacks homologues of either of these non-essential 80 α genes.

The cloned *dut* genes of 80 α and ϕ 11, as well as 80 α ORF15, complemented the respective phage deletion mutants when expressed under inducing conditions from the Pcad promoter in expression vector pCN51⁹ (Supplementary Tables 1 and 2). The cloned *dut* genes had no effect on SaPI induction by the phage mutants with defects in ORF15 or *sri* (data not shown). Similar complementation studies were not performed with 80 α *sri* because of the toxicity of this gene to *S. aureus*. An alternative approach to studies of Sri activity will be reported elsewhere (M. Harwich and G.E.C., unpublished data).

Expression of the cloned genes in SaPI-containing strains demonstrated that *dut* and ORF15 were sufficient to induce their respective SaPIs. As shown in Fig. 1b (lanes 1 and 3), when overexpressed, the cloned ϕ 11 and 80 α *dut* genes induced SaPIbov1 excision and replication. Similarly, plasmid-encoded 80 α ORF15 induced SaPIbov2 excision and replication (Supplementary Fig. 3). Finally, expression of 80 α ORF15 allowed high-frequency SaPIbov2 transfer by ϕ 11 (Supplementary Table 2), indicating that the absence of this gene in the ϕ 11 genome is the cause of its inability to induce SaPIbov2.

We next investigated the relationship between dUTPase activity and SaPIbov1 induction. The aspartate at position 81 in ϕ 11 Dut, predicted to be essential for activity¹⁰, was replaced with alanine, and the lack of dUTPase activity was confirmed *in vitro* (Supplementary Table 3). This mutant protein (D81A) retained wild-type SaPIbov1 induction activity (Fig. 1b, c, lane 2), indicating that dUTPase activity, per se, is not responsible for SaPIbov1 induction. The protein encoded by the ϕ 11 *dut* gene is required, however, as demonstrated by the lack of activity of a frameshift mutant (Fig. 1b, c, lane 5). Further evidence that the dUTPase and derepression activities are separate functions of the *dut* gene product was provided by one of the 80 α *dut* mutants that had been selected for resistance to SaPIbov1 interference. This mutant, a D95E substitution, retained dUTPase activity (Supplementary Table 3) even though it was defective for SaPI derepression (Supplementary Table 1), confirming that the derepression and dUTPase activities are separate. Thus, Dut represents a true ‘moonlighting’ protein with two different and genetically distinct activities.

We expected Dut-mediated derepression to involve interference with *stl* expression or function rather than Stl cleavage, as Stl lacks the consensus cleavage motif common to phage repressors, and SaPI induction does not involve the SOS response³ (see also Fig. 1c, lane 8). Using purified His-tagged proteins and a DNA probe consisting of the *stl-str* intergenic region, we showed first, by mobility shifts, that SaPIbov1 Stl binds to the site but ϕ 11 Dut does not (Fig. 2a). This indicates that Dut does not act by competing with Stl for access to its regulatory binding site. Because this fragment includes the *stl* promoter, Dut also cannot act as a repressor of *stl* expression. Addition of Dut blocked the Stl-mediated gel shift in a dose-dependent manner (Fig. 2a, right), suggesting that derepression involves Dut binding to Stl. This predicted protein–protein interaction was confirmed by coexpression and affinity purification of His₆–Stl_{SaPIbov1} and untagged Dut proteins. It was possible to co-purify a complex between His₆–Stl_{SaPIbov1} and Dut ϕ 11 (Fig. 2b, lane 1), whereas untagged Dut ϕ 11 alone did not bind to the resin (Fig. 2b, lane 2). Dut ϕ H15, which does not derepress SaPIbov1 (Fig. 1b), did not co-purify with His₆–Stl_{SaPIbov1} (lane 3), confirming the specificity of the Dut ϕ 11–His₆–Stl_{SaPIbov1} interaction. The identity of each of these bands was confirmed by amino acid sequencing and mass spectrometry. A similar interaction was observed with His-tagged 80 α ORF15 and SaPIbov2 Stl (Fig. 2b, lane 4), as well as with 80 α Sri and SaPI1 Stl (M. Harwich, A. Poliakov, J. Mobley and G.E.C., unpublished data), suggesting that the general mechanism of phage-induced SaPI derepression involves proteins that function as antirepressors, complexing with Stl to prevent it from binding to DNA.

If Dut acts by disrupting the binding of Stl to its target site, it should induce transcription of the Stl-repressed SaPI genes. This was confirmed using plasmid pJP674, which carries a β -lactamase reporter gene fused to *xis*, downstream of *str* and the Stl-repressed *str* promoter⁴, and also encodes Stl (see Fig. 2c). Cloned *dut* genes were introduced on vector pCN51 and expression was tested in the presence or absence of an inducing concentration of CdCl₂. Induction of ϕ 11 *dut*, but not PH15 *dut*, strongly increased β -lactamase expression from the *str* promoter (Fig. 2c). We conclude from these results that a SaPI-inducing Dut activates transcription by specifically disrupting the pre-formed Stl–DNA complex.

Insight into the possible domain involved in SaPIbov1 induction by Dut was provided by a comparison of predicted dUTPase sequences from various staphylococcal phages (Supplementary Fig. 2). This alignment showed high sequence similarity except for a central region of about 40 amino acids that was highly divergent among the *S. aureus* phage enzymes and was absent from the *S. epidermidis* phage PH15 dUTPase, which does not induce SaPIbov1 (Fig. 1b, c, lane 4 and Supplementary Table 1). Differential activity of the ϕ 11 and 80 α enzymes—which are fully conserved except for two residues in the amino-terminal region of the proteins and the divergent 40-aminoacid region, where they differ sharply (Fig. 3a)—suggested strongly that this region is involved in SaPIbov1 induction. The *dut* genes from these two phages had the same SaPIbov1 derepression activity when fully induced (Fig. 1b), but low constitutive expression of *dut*_{80 α in the absence of CdCl₂ failed to derepress SaPIbov1, although there was still full derepression by *dut* _{ϕ 11} under these conditions (Fig. 1c). Similar results in the absence of *dut* induction were seen in the complementation analysis reported in Supplementary Table 1. As the Dut protein levels produced from these constructs are comparable (Fig. 1c), the ϕ 11 Dut is more effective than that of 80 α in derepression of SaPIbov1. The difference in activity was mapped to the divergent region by exchanging the amino acids that differ between the ϕ 11 and 80 α Dut proteins and testing these derivatives for SaPIbov1 induction. Exchanging either of the two variable amino acids near the N terminus had no effect on derepression by either protein (Fig. 3b and Supplementary Table 1). However, when the divergent region was exchanged, the induction efficiency was transferred along with the exchanged amino acids (Fig. 3b and Supplementary Table 1). The possibility that differential expression of the two genes was responsible for the difference was ruled out by a western blot analysis (Fig. 3b), which confirmed that the two genes were expressed at the same levels.}

The absence of this central divergent region from the PH15 enzyme suggested that it might be an independent domain involved in relief of SaPIbov1 repression. However, deletion of this entire region from Dut _{ϕ 11} inactivated both dUTPase activity and SaPI induction (not shown). Furthermore, insertion of the divergent region from Dut _{ϕ 11} (N91–I128) between residues I91 and V102 of Dut_{PH15} did not confer induction activity on the chimaeric protein and also eliminated its dUTPase activity, indicating that this region is involved in the overall structure of the protein. Further mutants and structural analysis will be required to elucidate fully the dual functions of these dUTPases and their interaction with SaPIbov1 Stl.

A similar difference was found for derepressors of SaPIbov2. Phage 85 does not induce SaPIbov2, but it does encode a homologue of 80 α ORF15, designated ORF73 (Supplementary Fig. 4). Both 80 α ORF15 and ϕ 85 ORF73, when cloned in plasmid pCN51 and overexpressed, restored SaPIbov2 transfer in 80 α Δ ORF15 (Supplementary Table 2). However, with low basal expression, only 80 α ORF15 fully complemented SaPIbov2 transfer, indicating that these phages, too, carry allelic variants of inducing genes with different affinity for the SaPI-encoded repressors. Analysis of these allelic variants (in progress) is likely to be informative of the induction mechanism.

The process by which related SaPIs have acquired the ability to exploit entirely unrelated phage proteins as antirepressors represents a remarkable evolutionary adaptation. A single phage protein may have been originally targeted; because SaPIs interfere with phage maturation, mutational modification of such a protein to escape from SaPI derepression could have a selective advantage for the phage. A second stage in SaPI evolution could have involved divergence of the SaPI repressor, enabling it to complex with a different phage protein. More extensive analysis of SaPI derepression and the role of phage genes may clarify this and other interesting issues that have been identified in this study.

METHODS

DNA methods

Probes for detection of phage and SaPI DNA in Southern blots were generated by PCR using primers SaPIbov1-112mE and SaPIbov1-113cB (SaPIbov1), Sip-16mB and Sip-10cE (SaPIbov2), and Orf-24- ϕ 11-1mB and Orf-25- ϕ 11-1c (ϕ 11), which are listed in Supplementary Table 5. Labelling of the probes and DNA hybridization were performed according to the protocol supplied with the PCR-DIG DNA-labelling and chemiluminescent detection kit (Roche). Southern blots experiments were performed by standard procedures¹¹.

SaPI-resistant phage mutants were characterized by amplification of overlap-ping fragments by PCR and direct sequence analysis of gel-purified fragments. Sequencing was done by the Instituto de Biología Molecular y Celular de Plantas (IBMCP) and by Retrogen Inc.

Mobility shift assays

EMSA were performed as described before¹² using purified His₆-Stl or/and His₆-dUTPase proteins and a DIG-labelled DNA fragment obtained by PCR using the oligonucleotides 19-20upbov2 and 19-20 dwbov (listed in Supplementary Table 5). In each case purified dUTPase, Stl or both were added to the DNA-protein-binding mixture.

Enzyme assays

β -Lactamase assays, using nitrocefin as substrate, were performed with cells in exponential growth phase as described¹³, using a Thermomax (Molecular Devices) microtiter plate reader. β -Lactamase activities were recorded as initial slopes divided by cell density (maximum velocity (V_{max})/OD_{650nm}).

dUTPase activity was assayed using His₆-dUTPase proteins purified after expression in *E. coli*. Enzyme assays were performed using the EnzCheck Pyrophosphate Assay Kit (Molecular Probes), as previously reported¹⁴.

Plasmid construction

Plasmid constructs were prepared by cloning PCR products obtained with oligonucleotide primers as listed in Supplementary Table 5. All clones were sequenced by the Institute Core Sequencing Lab.

To introduce specific dut mutations into the phage, we used plasmid pMAD¹⁵ for allelic exchange as previously described^{3,4}.

In the western blot assays, probing was carried out with anti-33Flag antibodies (Sigma), according to the protocol supplied by the manufacturer.

In-gel enzymatic digestion and mass fingerprinting

Protein bands of interest were analysed as previously described⁶. Mass spectroscopy of proteins was performed by the ProteoRed Institute, at the Centro de Investigación Príncipe Felipe.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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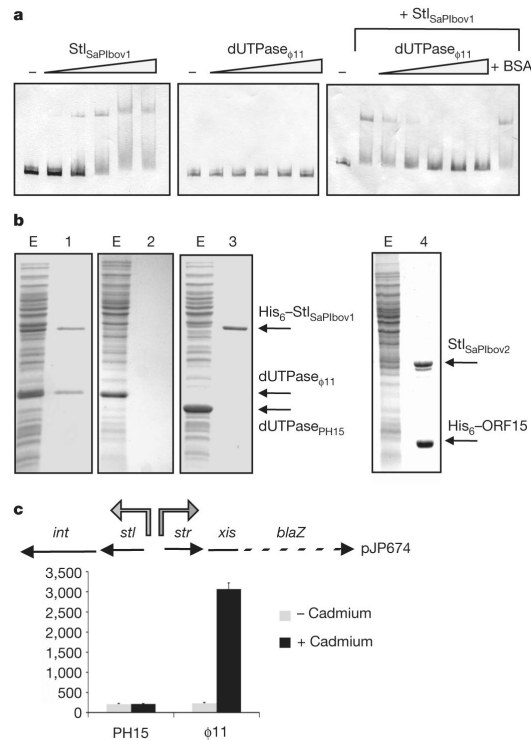


Figure 2. Phage-inducing proteins bind SaPI-encoded Stl proteins

a. dUTPase prevents Stl_{SaPIbov1} from binding to the *stl*–*str* divergent region. Shown are electrophoretic mobility shift assays in which increasing concentrations of Stl_{SaPIbov1} (0, 0.03, 0.06, 0.12, 0.24 and 0.48 μg; left), dUTPase_{φ11} (0, 0.02, 0.04, 0.08, 0.16 and 0.2 μg; middle), or Stl_{SaPIbov1} (0.12 μg) in the presence of dUTPase_{φ11} (0, 0.02, 0.04, 0.08, 0.16 and 0.2 μg) or 10 μg BSA (right) were mixed with labelled DNA containing the SaPIbov1 divergent region. **b.** Affinity chromatography of dUTPase using His₆-Stl_{SaPIbov1} (left), or affinity chromatography of Stl_{SaPIbov2} using His₆-ORF15_{φ80α} (right). *E. coli* strains expressing the different pairs were isopropyl-β-D-thiogalactoside (IPTG)-induced and, after disruption of the cells, the expressed proteins were applied to a Ni²⁺ agarose column and eluted. The presence of the different proteins was monitored in the load (lanes E), flow-through, wash and elute fractions by Coomassie staining. Elution fractions (lanes 1, 2, 3 and 4) were concentrated 2.5-fold relative to the load. Lane 1, His₆-Stl_{SaPIbov1} and dUTPase_{φ11} (JP6760); lane 2, dUTPase_{φ11} alone (JP6762); lane 3, His₆-Stl_{SaPIbov1} and dUTPase_{PH15} (JP6761); lane 4, His₆-ORF15_{φ80α} and Stl_{SaPIbov2} (JP6763). **c.** Derepression of *str* transcription by dut expression. Top, schematic representation of the *blaZ* transcriptional fusion generated in plasmid pJP674. Bottom, strains containing pJP674- and pCN51- derivative plasmids expressing dut_{PH15} (JP5469) or dut_{φ11} (JP5468) were assayed for β-lactamase activity in the absence of or 5 h after induction with 5 μM CdCl₂. Samples were normalized for total cell mass. Data are from an experiment in triplicate. Error bars represent s.d.

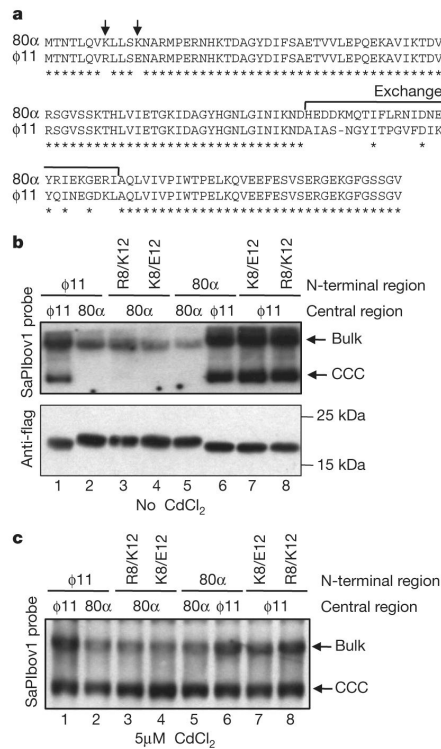


Figure 3. The level of SaPIbov1 inducing activity correlates with the central divergent region of Dut

a, Alignment of predicted staphylococcal phage dUTPase protein sequences from phages 80α and φ11. Black arrows indicate the two N-terminal variations between 80α and φ11. The bracket indicates the region that was exchanged between 80α and φ11 dUTPases. Asterisks indicate identical residues between phage 80α and φ11. **b, c**, SaPIbov induction and replication was measured in a non-lysogenic derivative of strain RN4220 carrying SaPIbov1 and plasmids expressing 3×flag-tagged Dut proteins containing substitutions of two amino acids in the N-terminal region or an exchange of the central region, as indicated. One millilitre of each culture ($OD_{540nm} = 0.3$) was collected in the absence of induction (**b**) or 3 h after treatment with 5 μM CdCl₂ (**c**) and used to prepare standard minilysates, which were resolved on a 0.7% agarose gel, blotted and probed for SaPIbov1 DNA or with antibody to the Flag tag. In both panels, lane 1, JP6789; lane 2, JP6794; lane 3, JP6793; lane 4, JP6800; lane 5, JP6797; lane 6, JP6795; lane 7, JP6798; lane 8, JP6799.

Table 1

Effects of phage mutations on SaPI replication and transfer

Phage	SaPIbov1		SaPIbov2		SaPII	
	Rpl	SaPI titre*	Rpl	SaPI titre*	Rpl	SaPI titre*
80α	+	4.1×10^7	+	1.3×10^8	+	1.6×10^8
80α Δ <i>dat</i>	2	8.3×10^2 [‡]	+	1.1×10^8	+	2.8×10^8
80α Δ <i>sri</i>	+	5.3×10^7	+	9.2×10^7	-	3.8×10^2 [‡]
80α ΔORF15	+	3.9×10^7	-	5.4×10^3 [‡]	+	4.1×10^8
φ11	+	1.8×10^7	-	ND	-	ND
φ11 Δ <i>dat</i>	-	9.2×10^2 [‡]	-	ND	-	ND

The means of results from three independent experiments are presented. Variation was within $\pm 5\%$ in all cases. Rpl, replication as determined by Southern blot. ND, not determined.

* Transductants ml^{-1} of lysate, using RN4220 as recipient.

[‡] Plaques ml^{-1} of lysate, using RN4220 as indicator.

[‡] This frequency is typical of transfer by generalized transduction and is not SaPI-specific.