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The type 2 dUTPase of bacteriophage ϕ NM1 initiates mobilization of *Staphylococcus aureus* bovine pathogenicity island 1

Rosanne L. L. Hill and Terje Dokland*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

Abstract

Staphylococcus aureus pathogenicity islands (SaPIs) are genetic elements that are mobilized by specific helper phages. The initial step in mobilization is the derepression of the SaPI by the interaction of a phage protein with the SaPI master repressor, Stl. Stl proteins are highly divergent between different SaPIs and respond to different phage-encoded derepressors. One such SaPI, SaPI_{bov1}, is derepressed by the dUTPase (Dut) of bacteriophage 80 α (Dut_{80 α}) and its phage ϕ 11 homolog, Dut₁₁. We previously showed that SaPI_{bov1} could also be mobilized by phage ϕ NM1, even though its *dut* gene is not homologous with that of 80 α . Here, we show that ϕ NM1 *dut* encodes a type 2 dUTPase (Dut_{NM1}), which has an α -helical structure that is distinct from the type 1 trimeric, β -sheet structure of Dut_{80 α} . Deletion of *dut*_{NM1} abolishes the ability of ϕ NM1 to mobilize SaPI_{bov1}. Like Dut_{80 α} , Dut_{NM1} forms a direct interaction with SaPI_{bov1} Stl both *in vivo* and *in vitro*, leading to inhibition of the dUTPase activity and Stl release from its target DNA. This work provides novel insights into the diverse mechanisms of genetic mobilization in *S. aureus*.

Graphical Abstract

Keywords

Molecular piracy; high frequency transduction; horizontal gene transfer; staphylococcal repressor; genetic regulation

*Corresponding author: Terje Dokland, Department of Microbiology, University of Alabama at Birmingham, 845 19th St South, BBRB 311, Birmingham, AL 35294, Tel: 205-9964502, dokland@uab.edu.

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Introduction

Staphylococcus aureus is a widespread commensal on human skin and a common colonizer of the nares, but is also an opportunistic pathogen involved in serious skin and systemic infections in humans and animals [1–3]. The emergence of highly virulent, community-acquired, methicillin-resistant *S. aureus* has become a major public health issue [4]. *S. aureus* is also one of the most common causes of chronic bovine mastitis, a considerable problem in the dairy industry [5, 6]. Most virulence determinants in *S. aureus* are encoded on mobile genetic elements (MGEs), such as bacteriophages and genomic islands, that allow the rapid evolution of the bacteria in response to selective pressures from the immune system or from subclinical doses of antibiotics [7, 8].

S. aureus pathogenicity islands (SaPIs) are MGEs, typically 15–20kb in size, that encode superantigen toxins and other virulence factors, and are commonly found in human and bovine pathogenic *S. aureus* strains [9, 10]. SaPI1 and SaPI2 were originally identified in the human menstrual toxic shock isolates RN4282 and RN3984, respectively [11, 12], while SaPIbov1 was isolated from bovine mastitis strain RF122 [13]. SaPIs are normally stably integrated in the host genome, but become mobilized at high frequency by specific “helper” phages [10, 14]. This results in the packaging of the SaPI genome into phage-like transducing particles, using structural gene products encoded by the helper phage, a process termed “molecular piracy” [14]. SaPIs encode several functions that interfere with phage replication, including a SaPI-specific small terminase subunit (TerS) [15] and proteins that redirect the phage assembly pathway to form capsids that are too small to package complete phage genomes [16–18]. These mechanisms contribute to the highly efficient horizontal transfer of virulence factors by SaPIs.

SaPIs are normally repressed through expression of its master repressor, Stl (Fig. 1), which is highly divergent between different SaPIs. In the presence of a compatible helper phage entering lytic cycle, Stl is derepressed by binding to a helper phage protein, leading to SaPI excision, replication, and interference with phage functions [14, 19, 20] (Fig. 1). Various early lytic genes of helper phage 80 α have been shown to derepress different SaPIs [20]: SaPI1 is derepressed by Sri, product of 80 α open reading frame (ORF) 22, SaPIbov1 is derepressed by Dut (ORF 32), and SaPIbov2 is derepressed by the product of ORF15 (gp15) [20]. Dut encodes a dUTPase, an enzyme that hydrolyzes dUTP to dUMP and is required by most cells to prevent the erroneous incorporation of dUTP into DNA [21]. The 80 α Dut protein (Dut_{80 α}) and its homolog in phage ϕ 11 (Dut₁₁) were shown to derepress SaPIbov1 by forming a direct interaction with Stl and preventing Stl from binding to DNA [20, 22, 23].

We originally identified the two phages ϕ NM1 and ϕ NM2 from the human clinical *S. aureus* strain Newman [24, 25] as likely helpers for SaPI1, based on the similarity of their Sri proteins to that of 80 α [26]. In contrast, the ORFs occupying the *dut* locus in ϕ NM1 and ϕ NM2 (ORF31 and ORF32, respectively) are extremely different from *dut*_{80 α} , corresponding to only 15% amino acid homology [26]. Unexpectedly, the phages were still able to mobilize SaPIbov1. (ϕ NM1 and ϕ NM2 share identical Dut protein sequences, and in the following we will refer only to ϕ NM1.)

Based on these observations, we asked whether the ϕ NM1 ORF31 indeed encoded a dUTPase, and if it was responsible for the mobilization of SaPIbov1, or if another ϕ NM1 gene was involved. Here, we show that ORF31 encodes a dUTPase (Dut_{NM1}) that belongs to the dimeric α -helical family of dUTPases, whereas Dut_{80 α} and Dut₁₁ have a trimeric β -sheet structure [22, 27]. Further, we show that no other ϕ NM1-encoded protein is involved in the derepression of SaPIbov1, and that there is a direct interaction between SaPIbov1 Stl and Dut_{NM1}. According to our model, interaction between Stl and Dut_{NM1} disrupts the Dut_{NM1} dimer and causes release of Stl from its DNA substrate.

Results

The *dut* gene of ϕ NM1 encodes a type 2 dUTPase

There are two classes of dUTPases. Type 1 dUTPases, the most common class, consist of an antiparallel β -sandwich and are usually trimeric [28–30]. The Dut proteins of 80 α and the closely related phage ϕ 11 belong to this class [22, 27] (Supplementary Fig. S1A). Type 2 dUTPases are far less common and consist of an α -helical dimer [31]. Only four structures of type 2 dUTPases have been determined, from *Campylobacter jejuni* [32], *Trypanosoma cruzi* [33], *Leishmania major* [34] and *Trypanosoma brucei* [35].

De novo modeling with I-TASSER [36] showed that Dut_{NM1} (178 residues, 20.7 kDa) belonged to the type 2 dUTPases, with greatest similarity to the dUTPase of *C. jejuni* (PDB ID: 1W2Y), with a C-score of 0.81 and a TM-score of 0.88 (Fig. 2; Supplementary Fig. S1B). The threading alignment generated by I-TASSER shows that the residues involved in dUTP hydrolysis and Mg²⁺ binding are conserved between Dut_{NM1} and the *C. jejuni* Dut (Fig. 2A). When the *C. jejuni* and predicted Dut_{NM1} structures are superimposed, it is clear that the core structure of the enzyme is conserved, with the major differences being the presence of two additional α -helices near the C-terminus and a longer loop between α 1 and α 2 in the *C. jejuni* enzyme (Fig. 2B).

Dut_{NM1} was cloned with a C-terminal His₆ tag, expressed in *E. coli* and purified by affinity chromatography. The predominantly α -helical nature of the protein was confirmed by circular dichroism (CD) spectroscopy. CDSSTR analysis of the CD data yielded an estimated 46% α -helical content with the remainder mostly turns and unordered regions (Supplementary Fig. S2). The predicted structure (Fig. 2B) has 70% α -helix (taking into account the His₆ tag), indicating that the protein may be partially disordered in solution.

Chemical crosslinking of Dut_{NM1} with paraformaldehyde resulted in a single band at $\approx$ 42 kDa, consistent with a Dut_{NM1} dimer (Fig. 3A). Glutaraldehyde crosslinking resulted in a single, diffuse band at $\approx$ 38 kDa (Fig. 3B). The smaller apparent mass with glutaraldehyde probably results from a more compact formation due to the stronger crosslinking agent.

To determine whether the purified Dut_{NM1} encoded a functional dUTPase, we employed an assay that measures the release of pyrophosphate (PP_i) upon dUTP hydrolysis. PP_i is detected spectrophotometrically by an increase in absorbance at 360 nm wavelength [37] (Fig. 4A,B; Supplementary Fig. S3A). As a positive control, we used Dut_{80 α} , for which the dUTPase activity was previously established [20] (Fig. 4A). Our results show that Dut_{NM1}

hydrolyzes dUTP with $V_{\max} = 0.18 \mu\text{M/s}$ and $K_M = 20.2 \mu\text{M}$; Fig. 4C), comparable to other members of the dimeric, α -helical pyrophosphohydrolase superfamily [31]. By comparison, the type 1 Dut₁₁ hydrolyzed dUTP with a $V_{\max} = 0.44 \mu\text{M/s}$, $K_M = 1.2 \mu\text{M}$ [22, 27].

Dut_{NM1} is responsible for derepression of SaPI_{bov1}

We showed previously that ϕNM1 (and its close relative ϕNM2) could mobilize SaPI_{bov1}, in spite of the difference in the ϕNM1 *dut* gene from that of 80 α [26]. We wanted to determine whether the derepression of SaPI_{bov1} by ϕNM1 was indeed dependent on Dut_{NM1} or whether another protein was involved in the process. Since the original ϕNM1 lysogen TB25 was made in the non-transformable parent strain RN450 [25], we first moved the phage into the transformable strain RN4220 [38, 39], yielding strain RH2. (For a list of bacterial strains and plasmids used in this study, see Tables 1 and 2.) We then used allelic exchange with the pMAD vector [40] to delete the *dut*_{NM1} gene from RH2, resulting in the phage ϕNM1 *dut* (strain RH3). Similarly, the 80 α *dut* gene was replaced with ϕNM1 *dut* in the 80 α lysogen RN10616 [41], yielding 80 α *dut*_{NM1} (strain RH4).

The 80 α and ϕNM1 wildtype (RN10616 and RH2) and mutant (RH3 and RH4) lysogens were then induced with mitomycin C, and the phage titers of the resulting lysates were assayed on RN4220. The mutant phage titers were similar to the wildtype, showing that *dut* is not essential for phage viability (Table 3). The phages were then used to infect the SaPI_{bov1} *tst::tetM*-containing strain JP45 [42], the SaPI1 *tst::tetM*-containing strain ST1 [43] and the SaPI2 *tst::tetM*-containing strain ST23 [43]. Transducing titers (cfu/ml) of the resulting lysates were determined by infecting RN4220 followed by plating on GL agar with the inclusion of 5 $\mu\text{g/ml}$ tetracycline. Phage titers (pfu/ml) were assayed by plating on RN4220 in the absence of antibiotic (Table 3; Fig 5).

Transducing titers for the 80 α and ϕNM1 wildtype phages on JP45 (SaPI_{bov1}) and ST1 (SaPI1) were comparable to those previously reported [26, 43] (Fig. 5A). ϕNM1 phage titers were greatly reduced on both JP45 and ST1, showing that SaPI1 and SaPI_{bov1} interfere strongly with phage production (Table 3; Fig. 5B). Deletion of *dut* from ϕNM1 abolished transduction of SaPI_{bov1} completely, and restored phage titers to normal. Substitution of *dut*_{80 α} with *dut*_{NM1} in 80 α did not affect the SaPI_{bov1} transduction frequency. These results show that Dut_{NM1} alone is responsible for the mobilization of SaPI_{bov1} by ϕNM1 , and that Dut_{NM1} and Dut_{80 α} are equally efficient at derepressing StI, in spite of their very different structures.

As expected, SaPI1 transduction, which depends on a different derepressor [20], was unaffected by deletion or substitution of *dut* (Fig. 5A). We also tested the mobilization of SaPI2, for which the derepressor has not been identified. SaPI2 mobilization by 80 α [43] was not affected by *dut* deletion or substitution (Fig. 5A). ϕNM1 mobilized SaPI2 with transduction frequencies at least three orders of magnitude less than for SaPI1 or SaPI_{bov1} (Fig. 5A), similar to generalized transduction rates [44].

Dut_{NM1} interacts directly with SaPIbov1 Stl

Previous studies had demonstrated that Dut_{80α} derepresses SaPIbov1 Stl through a direct interaction that disrupts the Stl DNA binding [20, 22]. To see whether Dut_{NM1} derepresses SaPIbov1 by a similar mechanism, we used a bacterial-two-hybrid (BACTH) assay to look for interactions between the two proteins. In this system, the two parts of adenylate cyclase (T18 and T25) are individually fused to the two potential binding partners. A positive interaction leads to reconstitution of the enzyme and cAMP production in an *E. coli* *cyc* host, which causes activation of *lac* and *mal* operons, which can be detected by a color change on specific media [45]. We generated constructs in which SaPIbov1 Stl was fused to either side of the T18 subunit, and Dut_{NM1} was fused to either side of the T25 subunit (Table 2). As a negative control, we used the empty vectors expressing the T18 and T25 subunits alone, and as a positive control, we used the plasmids pKT25-zip and pUT18C-zip that bring the two subunits together via a leucine zipper [45]. Pairs of plasmids were introduced into *E. coli* strain BTH101 and plated on MacConkey agar containing maltose, on which the color changes from white to red when the *mal* operon is expressed. Red colonies were observed when the plasmids pRH20 and pRH22, corresponding to Dut_{NM1} fused to the N-terminus of T25 (Dut_{NM1}-T25) and Stl fused to the N-terminus of T18 (Stl-T18), respectively (Table 2), were co-expressed (Fig 6A). This demonstrates that the two proteins interact *in vivo*. An interaction was also seen when Dut_{NM1} and Stl were placed at the C-termini of the T25 and T18 subunits (plasmids pRH17 and pRH23; data not shown), but not when Dut_{NM1} and Stl were on opposite sides, presumably because these orientations did not bring the T25 and T18 subunits into proximity.

To check for interaction *in vitro*, we co-expressed C-terminally His-tagged Dut_{NM1} with and without SaPIbov1 Stl in *E. coli* (plasmids pRH4 and pRH5; Table 2). The resulting clarified lysate was run over a Ni-NTA affinity column and eluted with 20–500 mM imidazole. The resulting eluate was analyzed by SDS-PAGE. As a control, we expressed His-tagged Dut_{80α} with and without SaPIbov1 Stl (plasmids pRH3 and pRH7), for which an interaction was demonstrated previously [20]. The results show that Dut_{NM1}-His and Dut_{80α}-His alone eluted at 150–250 mM imidazole (Fig 6B). When Dut_{NM1}-His and Stl (31.4 kDa) were co-expressed, however, bands corresponding to the two proteins co-eluted at 100–250 mM imidazole, showing that the two proteins were bound in solution (Fig 6B). As expected, Dut_{80α}-His and Stl also co-eluted at 150–250mM imidazole [20]. Untagged SaPIbov1 Stl alone did not bind to the affinity resin and came out during the 20 mM imidazole flow-through. These data show that Dut_{NM1}, like Dut_{80α} binds directly to SaPIbov1 Stl in spite of its different structure. The molar ratio of Stl:Dut_{NM1} based on the measured density of the bands by SDS-PAGE was 1:1.4, though this is likely to be an underestimate for Stl, since any free Dut_{NM1} protein would also bind to the affinity column. A band immediately below that corresponding to Dut_{NM1} always appears when it is bound to Stl, suggesting a conformational change leading to cleavage.

The Dut_{NM1}-Stl interaction was confirmed by chemical crosslinking experiments, which showed the formation of a complex with molecular weight of 50 kDa, indicative of a Dut_{NM1}-Stl heterodimer (predicted mass of 53.2 kDa) (Fig. 3C). As with Dut_{NM1} alone, the complex ran slightly smaller (≈43 kDa) than predicted when glutaraldehyde was used as the

crosslinker (Fig. 3D). We did not observe the Dut_{NM1} dimer band, nor bands corresponding to an Stl dimer or a (Dut_{NM1}–Stl)₂ heterotetramer, suggesting that Stl binding disrupts the Dut_{NM1} dimer.

The Dut_{NM1}–Stl complex was eluted from the affinity column and subjected to the same dUTPase assay as described for Dut_{NM1} (Fig. 4B,C; Supplementary Fig. S3B). The assay showed that the rate of dUTP hydrolysis by Dut_{NM1} is decreased in the presence of Stl, with a $V_{\max} = 0.08 \mu\text{M/s}$ and $K_M = 40.5 \mu\text{M}$ (Fig. 4B, C). Compared to the data for Dut_{NM1} alone ($V_{\max}=0.18 \mu\text{M/s}$, $K_M=20.2 \mu\text{M}$), these results indicate that Stl acts as a mixed inhibitor of Dut_{NM1}, consistent with the observed disruption of the Dut_{NM1} dimer.

Dut_{NM1} disrupts the binding of Stl to DNA

To determine whether Dut_{NM1} affects the DNA binding affinity of Stl, we used an electrophoretic mobility shift assay. A 170 bp DNA corresponding to the $P_{\text{stl}}\text{--}P_{\text{str}}$ promoter region (Fig. 1) of SaPIbov1 [20] was incubated with varying concentrations of Stl in the absence and presence of Dut_{NM1}, and separated by electrophoresis on a native acrylamide gel (Fig. 7). Under these conditions, the DNA itself formed two bands, one at the expected 170 bp size and one running anomalously at an apparent size of 850 bp. (Only one band was apparent by agarose gel electrophoresis.) Addition of 0.3–1.0 μM Stl led to a disappearance of the larger DNA band and a weakening of the DNA monomer band, concomitant with the appearance of a more slowly migrating band (Fig. 7). This demonstrated that there was an interaction between Stl and DNA leading to a shift in the DNA mobility, as previously described [20]. Addition of increasing concentrations of Dut_{NM1} led to a re-appearance of the DNA bands, and disappearance of the Stl–DNA band, showing that the Dut_{NM1} caused a release of Stl from the DNA substrate (Fig. 7). As a control, Dut_{NM1} alone did not cause a shift of the DNA bands, demonstrating the Dut_{NM1} does not have affinity for DNA. These results support a model for Dut_{NM1} derepression of SaPIbov1 that involves release of the Stl repressor from its target DNA, similar to that described for 80 α [20].

Discussion

We have shown in this paper that the type 2 dUTPase of ϕNM1 is equally effective at derepressing and mobilizing SaPIbov1 as the structurally distinct type 1 dUTPases of 80 α and ϕ11 . Like the type 1 dUTPases, Dut_{NM1} also forms a physical interaction with Stl that leads to its release from the target DNA [20, 23, 46]. Stl binding leads to inhibition of the dUTPase activity of Dut_{NM1}, similar to that described for Dut₁₁ [23].

Type 2 dUTPases are active in their dimeric form [31], which brings together residues from α_2 , α_3 and α_8 with the $\alpha_2\text{--}\alpha_3$ loop of the opposite subunit. (Supplementary Fig. S1B). Our crosslinking experiments showed that although Dut_{NM1} alone forms the expected homodimer, Dut_{NM1} and Stl form a heterodimer (Fig. 3C,D). This suggests that binding of Stl leads to disruption of the Dut_{NM1} dimer, consistent with the observed inhibition of dUTPase activity (Fig. 4C).

SaPIbov1 Stl has a predicted two-domain, α -helical structure with a helix-turn-helix (HTH) DNA binding motif near its N-terminus and a C-terminal domain that was shown to interact

with Dut₁₁ [46]. It forms dimers [23], analogous to other prokaryotic HTH repressors [47]. In our model, segregation of the Stl subunits through interaction with Dut_{NM1}, as suggested by our crosslinking experiments, leads to loss of DNA binding activity and consequent derepression of the *P_{str}* promoter (Fig. 8). Binding of the trimeric Dut_{80α} and Dut₁₁ proteins might similarly cause Stl segregation that leads to derepression of SaPIbov1 by phages 80α and φ11.

Tormo-Mas *et al.* [20, 22] and Szabo *et al.* [23] proposed different mechanisms by which Stl binding could be coupled to dUTP binding and hydrolysis. According to the Szabo *et al.* model, SaPI mobilization would only occur when the dUTP levels in the cells are low, ensuring a more fidelitous replication of the SaPI genome. While the dependence of Stl binding on Dut_{NM1} dUTPase activity is still unclear, our experiments indicate that Stl binding to Dut_{NM1} is not dependent on dUTP. The observed inhibition of dUTPase activity caused by Dut_{NM1} disruption could therefore be incidental to the mechanism of derepression in this system (Fig. 8).

Not all phage-encoded dUTPases can function as derepressors. For example, the type 1 dUTPase of *Staphylococcus epidermidis* phage PH15 (Dut_{PH15}) did not derepress SaPIbov1 [20], in spite of 43% sequence identity with Dut_{80α}. Conversely, other SaPIs are derepressed by phage proteins that are not dUTPases [20]. Sri, the 80α derepressor of SaPI1, is homologous to a phage protein known to bind the helicase loader DnaI and inhibit host DNA replication [43, 48]. It is not known whether binding to Stl inhibits Sri activity, but it is plausible that such inhibition might be relevant to ensure the fidelity of SaPI replication, similar to the role postulated for Dut₁₁ and SaPIbov1 [23]. Little is known about gp15, the derepressor of SaPIbov2. Other SaPIs, such as SaPI2, use still unidentified proteins for their derepression. It is unclear from this limited data whether there is a common theme between these different derepressors.

From the perspective of the SaPI, efficient derepression offers an escape mechanism by which it avoids being eliminated by a lytic phage [14]. The SaPI Stl repressors have evolved to sense the presence of a phage by interacting with a suitable phage protein that is expressed when the helper phage enters its lytic cycle. Like other derepressors that have been identified [20], the dUTPases of φNM1, 80α and φ11 are non-essential proteins that are expressed during the early phase of the phage's lytic cycle. Strikingly, Dut_{NM1} and Dut_{80α}/Dut₁₁, in spite of their different structures, are encoded by genes occupying the same locus in their respective phage genomes. It is still unclear if this is just a coincidence. However, the presence of these and other derepressors early in the lytic cycle could be important to ensure sufficient time for SaPI replication before packaging and cell lysis.

Genetic mobilization is key in the ever rising diversity of microorganisms. Transduction by phages is the main mechanism of horizontal gene transfer in staphylococci, which are not naturally transformable [49]. Phages are responsible for both generalized transduction of plasmid and chromosomal DNA, and high frequency specialized transduction of SaPIs [50]. The acquisition of SaPIs is central to the evolution of increased virulence in community-acquired *S. aureus* [51]. SaPIs carrying virulence genes have been transferred to other bacterial species, even across genera, under conditions that mimic the natural environment

[52]. More detailed information on the processes involved in SaPI derepression and mobilization will be important for a better understanding of genetic mobilization mechanisms in *S. aureus* and other human pathogens, and may eventually lead to novel approaches for the treatment of important human diseases.

4. Methods

Growth of bacteria and phages

Strains used in this study are listed in Table 1. *S. aureus* lysogens were grown in CY medium at 32 °C until the absorption at 600 nm wavelength (A_{600}) reached an O.D. of 0.6, upon which the prophages were induced by the addition of 2.5 µg/ml mitomycin C [53]. Cells were grown until lysis occurred, as evidenced by a drop in A_{600} , typically after 3–4 h. The lysates were clarified by centrifugation at 7,500g, 20 min. The resulting supernatants were filtered through a 0.22 µm filter to remove remaining intact cells and checked by negative stain EM (UAB cryo-EM core) for the presence of phage. Phage titers were measured by plating serial dilutions on a lawn of RN4220 on phage agar [53]. The ϕ NM1 lysogen RH2 was made by infecting RN4220 with a lysate from induction of TB25 at an m.o.i = 5, and plating on GL agar.

Cloning and allelic exchange

PCR primers were supplied by Eurofins MWG Operon Inc., Huntsville, AL. PCR was performed using an Accuprime Pfx kit (Life Technologies, Carlsbad, CA). Allelic exchange was carried out using the pMAD vector, as previously described [17, 40]. To generate ϕ NM1 *dut*, an insert that included 400–700 nucleotides flanking the *dut*_{NM1} gene was generated by PCR and cloned into pMAD, generating plasmid pRH15 (Table 2). To generate 80 α *dut*_{NM1}, the *dut*_{NM1} gene flanked by 400–700 nucleotides from each side of *dut*_{80 α} was cloned into pMAD, generating plasmid pRH14. pRH15 and pRH14 were transformed by electroporation into RH2 and the 80 α lysogen RN10616, respectively (Tables 1 and 2). The resulting cells were plated on TSA with 5 µg/ml erythromycin and 200 µg/ml X-gal, at 44 °C. Blue colonies, indicating integration of the plasmid, were sub-cultured at 30 °C and 44 °C and selected for erythromycin sensitivity and white colonies on TSA, indicating loss of the vector backbone. The resulting lysogens were induced by mitomycin C and checked by EM and sequencing (UAB Heflin center genomics core) of the recombinant phage genomes.

Transduction assays

For phage-induced SaPI mobilization, 4 ml cultures of the appropriate SaPI-containing strains (ST1, ST23 or JP45) were grown in CY at 32 °C in the presence of 5µg/ml tetracycline. At A_{600} = 0.3, the cells were infected at an approximate m.o.i. = 1 with the filtered phage lysates. The resulting lysates were collected after 4 h and filtered. Serial dilutions (100 µl volume) were mixed with equal volumes of RN4220 cells, incubated for 30 min at 32 °C, and spread on GL agar plates containing 5 µg/ml tetracycline. The plates were incubated at 32 °C for 48 h and colonies were counted. Phage titers were calculated as above.

Purification of Dut

Expression constructs containing the coding sequences for Dut_{80α} or Dut_{NM1}, including a C-terminal His₆ tag were cloned alone or in combination with SaPIbov1 Stl into the vector pET21a using the *E. coli* cloning strain DH5α (Tables 1 and 2). The plasmids were transformed into the *E. coli* expression strain BL21 Star (DE3) pLysS (Novagen), grown in LB at 37°C, and induced with 0.5 mM IPTG at A₆₀₀=0.6. After 3 h of growth at 37 °C, the cells were harvested by centrifugation at 6,000g for 10 min and resuspended in lysis buffer (100 mM Tris-HCl pH 7.4, 200mM NaCl). 60 units/ml BenzonaseTM nuclease (Novagen) and 20 µg/ml lysozyme (GoldBioCom, St. Louis, MO) were added, cells incubated for 30 min at 37 °C, followed by three passages through a high pressure cell disruptor (Avestin Inc., Ottawa). After centrifugation at 17,000g, 30 min, the supernatant was passed over an 8 ml Ni-NTA affinity column (Bio-Rad, Hercules, CA), and eluted with 20–500 mM imidazole. The protein samples (30 µl) were separated by SDS-PAGE on 4–15% gradient gels, and stained with 1% Coomassie brilliant blue.

dUTPase assay

dUTPase enzyme activity assays were performed using the EnzCheck Pyrophosphate Assay Kit (Molecular Probes, Eugene, OR) that measures the release of PP_i upon dUTP hydrolysis by its reaction with 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) in the presence of purine nucleoside phosphorylase (PNP). The resulting product 2-amino-6-mercapto-7-methylpurine is detected by absorbance at 360 nm wavelength [37]. For this assay, we added 0–60 µM dUTP to 46 nM Dut_{NM1}-His or 65 nM Dut_{80α}-His and followed the absorbance at 360 nm for 30 min at 22 °C in a Beckman DU-640 spectrophotometer, compared to a standard curve with known quantities of PP_i. All reactions were carried out in triplicate. V₀ values were calculated from the linear portion of the graphs and plotted on a Lineweaver-Burk plot as 1/V₀ against the reciprocal of the substrate concentration to obtain V_{max} and K_M for the reactions.

Chemical crosslinking

Affinity-purified Dut_{NM1} alone or the Dut_{NM1}-Stl complex, at concentrations of 0.35–1.6 mg/ml, were crosslinked with 2% paraformaldehyde or 0.01% glutaraldehyde for up to 30 min at 4°C in 100 mM Na phosphate buffer pH 7.8 with 600 mM NaCl, 0.15% Tween-20, 5 mM MgCl₂. At specific time points, 20 µl aliquots were taken out and the reaction was stopped by the addition of 5 µl 1M Tris pH 8.0. The resulting mixture was separated by 4–15% SDS-PAGE and Coomassie stained.

Electrophoretic mobility shift assay

To obtain purified Stl protein, the SaPIbov1 Stl coding sequence was cloned into the vector pGEX-6P-1 in-frame with the GST sequence. The protein was expressed in BL21 Star (DE3) cells, lysed by addition of lysozyme, freeze-thawing and sonication, and purified by batch affinity on glutathione-sepharose beads (Amersham Biosciences). The GST tag was removed by overnight cleavage with PreScission protease (GE Health Sciences), while on the beads.

A 170 bp DNA fragment consisting of the SaPI_{bov1} P_{stl} – P_{str} promoter region was generated by PCR using the primers described by Tormo-Mas et al. 2010 [20]. 36–75 ng of DNA were combined with 0.3–1.0 μ M StI (final concentration) and 0–6.4 μ M Dut_{NM1} and incubated at 22 °C for 1 h before separation on Novex™ 4–20 % native Tris-Glycine gels (Life Technologies, Carlsbad, CA). The gels were stained with 0.15 μ g/ml ethidium bromide and visualized under UV light.

Bacterial-two-hybrid assay

The full-length Dut_{NM1} coding sequence was cloned into the XbaI and KpnI sites of plasmids pKT25 or pKNT25 for C-terminal or N-terminal fusion to the T25 domain of adenylate cyclase [45]. Similarly, SaPI_{bov1} StI was cloned into the XbaI and KpnI sites of plasmids pUT18 and pUT18C for fusion to the T18 domain of adenylate cyclase. The plasmids were co-transformed into *E. coli* strain BTH101 and plated on MacConkey agar with 1% maltose in the presence of 100 μ g/ml ampicillin and 50 μ g/ml kanamycin. Empty vectors, expressing only the two adenylate cyclase domains, were used as a negative control. Vectors expressing the two parts of a leucine zipper fused to T25 and T18 were used as a positive control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *S. aureus* pathogenicity islands (SaPIs) are mobilized through derepression by phage proteins
- SaPI_{bov1} is derepressed by phage ϕ NM1 Dut, a type 2 α -helical dUTPase (Dut_{NM1})
- Dut_{NM1} interacts with the SaPI_{bov1} Stl repressor, releasing it from its DNA target
- Interaction with Stl leads to inhibition of the dUTPase activity of Dut_{NM1}
- This work suggests a novel mechanism for the derepression that leads to SaPI mobilization

Figure 1.

Schematic diagram of the *str* and *stl* operons of SaPIbov1, showing the integrase (*int*, light blue), master repressor (*stl*, red), transcriptional activator (*str*, green), and excisionase (*xis*, yellow) genes (drawn to scale). The P_{stl} , P_{str} and presumptive P_{int} promoters are indicated. The repression of P_{str} by Stl is shown by the red bar-headed arrow, and the derepression of Stl by a phage protein by the green bar-headed arrow.

Figure 2.

Structure prediction of Dut_{NM1}. (A) Threading alignment of the dUTPase sequence from phage ϕ NM1 (fNM1_Dut) with that from *C. jejuni* (CjeJ_Dut; PDB ID: 1W2Y), generated by I-TASSER [36]. Identical residues are highlighted on a red background, conserved residues in boxed red text. Secondary structure elements in 1W2Y are shown above the sequence. Residues involved in Mg²⁺ binding are indicated by circles; other active site residues by diamonds. Figure generated with ESPript [54]. (B) Ribbon representation showing the superposition of the I-TASSER prediction of Dut_{NM1} (orange) on one subunit of the *C. jejuni* Dut structure (blue). The Dut_{NM1} active site residues (F50, K51, W53 and K159) are shown as purple stick representations.

Figure 3.

Crosslinking of Dut_{NM1} and SaPIbov1 Stl. (A, B) Purified Dut_{NM1} crosslinked with 2% paraformaldehyde (A) or 0.01% glutaraldehyde (B) for the indicated times (minutes), separated by SDS-PAGE. Lane U, no crosslinker. Bands corresponding to Dut monomer and Dut₂ dimers are indicated. The band indicated Dut* corresponds to a presumed compact, crosslinked Dut monomer. (C, D) Affinity co-purified Dut_{NM1} and Stl crosslinked with 2% paraformaldehyde (C) or 0.01% glutaraldehyde (D), as above. The bands corresponding to Dut, Stl and the Dut-Stl heterodimer are indicated. M, markers, molecular weights (kDa) indicated.

Figure 4.

dUTPase activity of Dut_{NM1}. (A) Detection of pyrophosphate (PP_i) release after 15 min of dUTP hydrolysis by Dut_{NM1} and Dut_{80α}. (+) indicates that 20μM dUTP was added; (-) is control without dUTP. 20 μM PP_i was used as a positive control. Average of three replicates; standard deviation shown. (B) Time course of dUTP hydrolysis by Dut_{NM1} and the Dut_{NM1}-Stl complex for 20 μM and 40 μM dUTP substrate concentrations, measured as PP_i release. Average and standard deviation of three replicates are shown. (C) Lineweaver-Burk plots for the dUTPase activity of Dut_{NM1} (blue diamonds) and the Dut_{NM1}-Stl complex (red squares).

Figure 5.

Transduction by phages 80α and φNM1. *S. aureus* strains containing SaPI1 *tst::tetM* (ST1; red), SaPI2 *tst::tetM* (ST23; blue) and SaPIbov1 *tst::tetM* (JP45; purple) (Table 1) were infected with the indicated phages. (A) The resulting lysates were used to infect RN4220 and plated in the presence of tetracycline for calculation of transducing titers (cfu/ml). (B) Phage titers (pfu/ml) of the resulting lysates, assayed on RN4220. The bars represent the average of three experiments; standard deviations are indicated.

Figure 6.

Interaction between Dut_{NM1} and Stl. (A) Bacterial-two-hybrid analysis. The spots represent individual colonies on the same plate; three independent colonies for each combination are shown (columns 1–3). Top row: The positive control plasmids pKT25-zip and pUT18C-zip expressing the two adenylate subunits T25 and T18 with a leucine zipper; Middle: Negative control plasmids pKNT25 and pUT18 expressing only the two adenylate cyclase subunits; Bottom: Expression of the Dut_{NM1}-T25 fusion (plasmid pRH20) and T18-Stl fusion (pRH22). A deep red color indicates a positive interaction. (B) Coomassie stained 4–15% SDS-PAGE of the 200 mM imidazole eluate from a Ni-NTA affinity column, showing expression of Dut_{NM1}-His alone, Dut_{NM1}-His+Stl co-expression, Dut_{80α}-His alone, Dut_{80α}-His+Stl co-expression, and Stl alone. M, marker, molecular weights (kDa) indicated.

Figure 7.

Electrophoretic mobility shift assay. A 170 bp PCR product corresponding to the P_{stl} - P_{str} promoter region of SaPIbov1 was incubated with the indicated concentrations (μM) of Stl and/or Dut_{NM1} and separated on a native 4–20% polyacrylamide gel. The gel was stained with ethidium bromide and visualized under UV light. The density was inverted for clarity. M, marker; size (in bp) indicated for pertinent bands. The PCR product formed two bands under these conditions; one at the expected 170 bp size (DNA) and a band (DNA*) that ran anomalously at a larger apparent size. The arrow indicates the DNA-Stl complex band.

Figure 8.

Model for derepression by Dut_{NM1} . In the repressed state, the Stl repressor (red triangles) prevents transcription from the P_{str} promoter. Upon derepression, binding of Dut_{NM1} (purple semicircles) leads to separation of both the Dut_{NM1} and Stl dimers, blocking the dUTPase activity of Dut_{NM1} and releasing Stl from the P_{str} promoter, ultimately leading to SaPI mobilization.

Table 1

List of strains used in this study.

Strain	Description	Reference
<i>E. coli</i> strains:		
DH5 α	Recombination-deficient cloning strain	Novagen
BL21 Star (DE3)	Expression strain, increased RNA stability	Novagen
BTH101	Adenylate cyclase deficient strain for BACTH	[45]
<i>S. aureus</i> strains:		
RN4220	RN450, mutated to accept foreign DNA	[39]
RN10616	RN4220 (80 α)	[41]
TB25	RN450 (ϕ NM1)	[25]
ST1 [§]	RN4220 (SaPI1 <i>tst::tetM</i>)	[43]
JP45	RN4220 (SaPIbov1 <i>tst::tetM</i>)	[42]
ST23 [†]	RN4220 (SaPI2 <i>tst::tetM</i>)	[43]
RH2	RN4220 (ϕ NM1)	This study
RH3	RN4220 (ϕ NM1 <i>dut</i>)	This study
RH4	RN4220 (80 α <i>dut</i> _{NM1})	This study

[§]Independent isolate, identical to strain RN10822 [43].

[†]Independent isolate, identical to strain RN10823 [43].

Table 2

Plasmids used in this study

Plasmid	Description	Reference
pET21a	<i>E. coli</i> expression vector, Amp resistance	Novagen
pGEX-6P-1	<i>E. coli</i> expression vector, Amp resistance	GE Healthcare Life Sciences
pMAD	Allelic exchange shuttle vector, Amp/Erm resistance	[40]
pKT25	BACTH vector, <i>cya</i> T25 subunit, C-terminal insert, Kan resistance	[45]
pNKT25	BACTH vector, <i>cya</i> T25 subunit, N-terminal insert, Kan resistance	[45]
pUT18	BACTH vector, <i>cya</i> T18 subunit, N-terminal insert, Amp resistance	[45]
pUT18C	BACTH vector, <i>cya</i> T18 subunit, C-terminal insert, Amp resistance	[45]
pKT25- <i>zip</i>	pKT25 <i>cya</i> T25 with C-terminal Leu zipper	[45]
pUT18C- <i>zip</i>	pUT18C, <i>cya</i> T18 with C-terminal Leu zipper	[45]
pRH3	pET21a <i>dut</i> _{80a} -His ₆	This study
pRH4	pET21a <i>dut</i> _{NM1} -His ₆	This study
pRH5	pET21a <i>dut</i> _{NM1} -His ₆ , <i>stl</i>	This study
pRH6	pET21a <i>stl</i>	This study
pRH7	pET21a <i>dut</i> _{80a} -His ₆ , <i>stl</i>	This study
pRH14	pMAD for replacement of <i>dut</i> _{80a} by <i>dut</i> _{NM1}	This study
pRH15	pMAD for deletion of <i>dut</i> _{NM1}	This study
pRH17	pKT25 <i>cya</i> T25- <i>dut</i> _{NM1} fusion	This study
pRH20	pNKT25 <i>dut</i> _{NM1} - <i>cya</i> T25 fusion	This study
pRH22	pUT18 <i>stl</i> - <i>cya</i> T18	This study
pRH23	pUT18C <i>cya</i> T18- <i>stl</i>	This study
pRH24	pGEX-6P <i>stl</i>	This study

Table 3

Transducing and phage titers.

Bacteriophage	Phage titer [¶] (pfu/ml)	Transducing titers/phage titers (cfu/pfu) [§]		
		ST1 (SaPI1)	ST23 (SaPI2)	JP45 (SaPIbov1)
80α	1.2×10^8	2.5×10^{-2}	8.1×10^{-2}	1.3×10^{-1}
φNM1	1.0×10^8	3.2×10^1	1.1×10^{-6}	1.4×10^1
80α <i>dut</i> _{NM1}	7.0×10^8	6.6×10^{-2}	1.4×10^{-1}	6.3×10^{-1}
φNM1 <i>dut</i>	3.0×10^8	8.3×10^{-3}	2.8×10^{-6}	4.8×10^{-8}

[¶]Titers of the phage used for the transduction, plaque forming units per ml on RN4220

[§]Transducing titers (tetracycline resistant colony forming units per ml of lysate), divided by phage titers (pfu/ml), using RN4220 as recipient. All SaPIs also include the *tetM* marker gene.