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Derepression of SaPI_{bov1} is independent of ϕ NM1 type 2 dUTPase activity and is inhibited by dUTP and dUMP

Rosanne L. L. Hill¹, Jiri Vlach¹, Laura K. Parker², Gail E. Christie², Jamil S. Saad¹, and Terje Dokland^{1,*}

¹Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294, USA

²Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA 23298, USA

Abstract

Staphylococcus aureus is an opportunistic human pathogen able to transfer virulence genes to other cells through the mobilization of *S. aureus* pathogenicity islands (SaPIs). SaPIs are derepressed and packaged into phage-like transducing particles by helper phages like 80 α or ϕ NM1. Phages 80 α and ϕ NM1 encode structurally distinct dUTPases, Dut_{80 α} (type 1) and Dut_{NM1} (type 2). Both dUTPases can interact with the SaPI_{bov1} Stl master repressor, leading to derepression and mobilization. That two structurally distinct dUTPases bind the same repressor led us to speculate that dUTPase activity may be important to the derepression process. In type 1 dUTPases, Stl binding is inhibited by dUTP. The purpose of this study was to assess the involvement of dUTP binding and dUTPase activity in derepression by Dut_{NM1}. Dut_{NM1} activity mutants were created and tested for dUTPase activity using a novel NMR-based assay. We found that all Dut_{NM1} null activity mutants interacted with the SaPI_{bov1} Stl C-terminal domain, formed Dut_{NM1}–Stl heterodimers, and caused release of the *P*_{str} promoter. However, promoter release was inhibited in the presence of dUTP or dUMP. We tested two ϕ NM1 mutant phages that had null enzyme activity and found they could still mobilize SaPI_{bov1}. These results show that only the *apo* form of Dut_{NM1} is active in Stl derepression, and that dUTPase activity is not necessary for mobilization of SaPI_{bov1} by Dut_{NM1}.

Graphical abstract

*Corresponding author. Terje Dokland, Department of Microbiology, University of Alabama at Birmingham, 845 19th St South, BBRB 311, Birmingham, AL 35294, Tel: 205-996-4502, dokland@uab.edu.

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Keywords

bacteriophage; *Staphylococcus aureus* pathogenicity island; molecular piracy; staphylococcal repressor; nuclear magnetic resonance (NMR)

Introduction

Staphylococcus aureus is a Gram positive opportunistic bacterium that is occasionally implicated in serious infections [1–3]. About 20–30% of people carry *S. aureus* in their nares without disease, including strains that are resistant to methicillin and other antibiotics [4]. People colonized with *S. aureus* are more susceptible to infection, which occurs when there is a breach of the skin or mucosal membrane [3, 5–7]. Pathogenic *S. aureus* isolates have an up-regulation of virulence genes compared to the commensal strains [8, 9].

Virulence genes in *S. aureus* are typically carried on mobile genetic elements that are transferred horizontally between bacteria. *S. aureus* pathogenicity islands (SaPIs) are mobile genetic elements that carry genes encoding virulence factors such as superantigen toxins, adhesins, anticoagulants, and immunomodulatory factors [10–14]. For example, several SaPIs, including SaPI1, SaPI2, and SaPIbov1, contain the *tst* gene encoding toxic shock syndrome toxin (TSST-1) [15, 16], which was identified as the cause of toxic shock syndrome outbreaks in the 1980s [17]. SaPIbov1 was identified in the bovine mastitis *S. aureus* strain RF122, and also encodes staphylococcal enterotoxin genes *sec* and *seI* [18].

SaPIs are mobilized by specific “helper” phages at high frequency, leading to packaging of the SaPI genomes into phage-like transducing particles using structural proteins encoded by the helper [10, 12]. The helper phages initiate SaPI mobilization by producing proteins that interact with the SaPI master repressor, Stl, leading to derepression and expression of genes involved in SaPI excision and replication [19]. SaPIbov1 was shown to be derepressed by the type 1 dUTPase encoded on phages 80α and φ11 (Dut_{80α} and Dut₁₁ respectively) [19, 20]. We showed previously that SaPIbov1 could also be derepressed by the type 2 dUTPase

from phage ϕ NM1 (Dut_{NM1}) [21, 22]. Type 1 dUTPases have a β -pleated jellyroll fold and are primarily trimers [20, 23–25], while type 2 dUTPases are α -helical dimers [26–28]. In spite of their different structures, both classes of dUTPases hydrolyze dUTP to dUMP [23, 27, 29].

Interaction of the helper phage-encoded dUTPases with Stl causes Stl to release repression of the P_{str} promoter, leading to excision and replication of the SaPI element [19, 21, 30–34]. Stl is an α -helical protein composed of an N-terminal DNA binding domain with two key residues (Q40 and N41) responsible for binding DNA, and a C-terminal domain that was shown to interact with type 1 dUTPases [35]. Binding of the type 1 Dut_{80 α} and Dut₁₁ is inhibited by dUTP, and its dUTPase activity is inhibited by Stl binding [25, 34, 36]. We showed previously that the presence of Stl inhibited dUTPase activity of type 2 Dut_{NM1} by forming a Dut_{NM1}-Stl heterodimer that led to the release of the target DNA [21]. However, the actual mechanism of SaPI derepression is still not clear. Is derepression coupled to dUTP binding and hydrolysis in type 2 dUTPases, like Dut_{NM1}? Does Dut_{NM1}, like its type 1 dUTPase counterparts, interact with the C-terminal domain of Stl or directly block the DNA-binding N-terminal domain?

In this study, we sought to answer these questions. First, we created mutations in the predicted active site residues of Dut_{NM1} to identify key residues involved in dUTP hydrolysis. The mutants were tested for enzyme activity using a novel nuclear magnetic resonance (NMR) based assay. Wildtype (WT) Dut_{NM1} and mutants were also tested for their ability to bind Stl and to cause release of the P_{str} DNA in the absence and presence of dUTP and dUMP. We also created truncations of Stl to determine if Dut_{NM1} interacts with the same Stl domain as type 1 dUTPases. Finally, the mutant *dut* genes were introduced into a ϕ NM1 lysogen to check for SaPI_{bov1} mobilization. We show that dUTPase activity of Dut_{NM1} is not required for interaction with Stl and SaPI_{bov1} mobilization. Instead, the derepression is inhibited by high concentrations of dUTP and dUMP. We also show that Dut_{NM1} interacts with the C-terminal domain of Stl. Together our data provide new insights into the derepression and mobilization mechanism of SaPI_{bov1}.

Results

Mutation of active site residues of Dut_{NM1}

Several amino acid residues predicted to play a role in the dUTPase activity of Dut_{NM1} were identified by comparison with the crystal structures of the homologous type 2 dUTPases of *Leishmania major*, *Trypanosoma cruzi*, and *Campylobacter jejuni* [26, 28, 37]. The predicted structure of Dut_{NM1} is most similar to the *C. jejuni* enzyme (PDB ID: 1W2Y; Fig. 1A) [21]. Type 2 dUTPases are dimers with two active sites located at the dimer interface (Fig. 1B). The predicted catalytic residues include E39, E42, E67 and D70, which comprise two Mg²⁺ binding sites, and K159 on one subunit; and F50, K51, and W53, located in the α 2– α 3 loop in the second subunit (Table 1; Fig. 1). The active site residues are completely conserved between Dut_{NM1} and the *C. jejuni* enzyme (Fig. 1A). Based on this prediction, we generated five different plasmid clones expressing Dut_{NM1} proteins with mutations expected to abolish dUTPase activity (Tables 1 and 2): Dut_N^{K159} (K159A), Dut_N^{FKW} (F50A, K51A, W53A), Dut_N^{MgA} (E67A, D70A), Dut_N^{MgB} (E39A, E42A) and Dut_N^{MgAB} (both Mg²⁺

binding sites). All Dut_{NM1} mutant constructs had C-terminal His₆ tags, which were used for nickel affinity purification.

The Dut_{NM1} mutant proteins were checked by circular dichroism (CD) spectroscopy to determine whether the mutations had any adverse effects on the secondary structure of the proteins. The α -helical content for WT Dut_{NM1} based on the predicted structure is 55% whereas the calculated value (using Selecon-3) based on the CD spectrum was 44%. All mutant proteins exhibited characteristic negative peaks at 208 and 222 nm wavelength, consistent with α -helical organization (Fig. 2A). However, some differences existed in the calculated α -helical content compared to WT Dut_{NM1}. Dut_N^{FKW} displayed a gain of α -helical content, perhaps due to stabilization of the α 2– α 3 loop, whereas the other mutants displayed a net loss of α -helical content (Table 1; Fig. 2A), perhaps due to loss of Mg²⁺ [38, 39]. However, CD spectrum analysis is highly reliant on protein concentration, and since some of the mutants were prone to aggregation (most notably Dut_N^{MgB} and Dut_N^{MgAB}, which displayed the greatest loss of α -helical structure), the determined concentrations may not have been accurate. The Dut_{NM1} mutants were also subjected to crosslinking with paraformaldehyde to see if they retained the ability to form dimers. All Dut_{NM1} mutants, after crosslinking and separation on SDS-PAGE, developed bands at $\approx$ 40 kDa, consistent with a dimer (Fig. 2B).

To determine if the mutant proteins retained dUTPase activity, we employed a novel one-dimensional ¹H-NMR spectroscopic assay to detect and monitor dUTP hydrolysis. Firstly, ¹H chemical shifts of free dUTP and dUMP were assigned as described in materials and methods (Supplementary Fig. S1). In the absence of enzyme, dUTP was stable for at least a month under the conditions used for the assay (data not shown). We monitored the hydrolysis of dUTP using H-4', H-5', and H-6 signals, which do not overlap in the mixture of dUTP and dUMP (Supplementary Fig. S1). Under these conditions, WT Dut_{NM1} at 200 nM was able to fully hydrolyze 1 mM dUTP in approximately 2hrs (Fig. 3). All null activity mutants failed to hydrolyze 1 mM dUTP in 24hrs. For Dut_N^{K159} and Dut_N^{MgA}, it took approximately two weeks for complete hydrolysis of 1 mM dUTP (Fig. 3). The other mutants failed to completely hydrolyze 1 mM dUTP even after two weeks (Fig. 3). The NMR results were consistent with a pyrophosphate detection assay, which indicated significant reduction of the hydrolysis activity for the mutants compared to WT Dut_{NM1} (Supplementary Fig. S2). The pyrophosphate detection assay could not be used to follow the reaction beyond a few hours, due to limited stability of the reagents at room temperature [40].

dUTPase activity mutants interact with Stl

We have shown previously that Dut_{NM1} interacts with Stl both *in vitro* and *in vivo* [21]. We have also shown through crosslinking experiments that Dut_{NM1} and Stl form a $\approx$ 50 kDa heterodimer [21]. To determine if the Dut_{NM1} activity mutants affected Stl binding, we mixed the two purified proteins at a Dut_{NM1}:Stl molar ratio of 4:1 and incubated for 1hr, followed by crosslinking with 2% paraformaldehyde. When the crosslinked proteins were separated by SDS-PAGE, $\approx$ 40 kDa and $\approx$ 50 kDa bands were observed (Fig. 4A). The 40 kDa band is consistent with the previously shown Dut_{NM1} homodimer [21], formed because

Dut_{NM1} was in excess. The 50 kDa band is consistent with a Dut_{NM1}-Stl heterodimer [21]. These results show that the Dut_{NM1} mutations did not affect binding to Stl.

We showed previously that the interaction between WT Dut_{NM1} and Stl caused Stl to release from the *P_{str}* DNA, similar to what was observed for Dut_{80α} and Dut₁₁ [19–21]. We used an electrophoretic mobility shift assay (EMSA) to test whether the Dut_{NM1} null activity mutants would also cause the release of DNA upon interaction with Stl. Incubation of Stl with the 57bp promoter region from *P_{str}* caused an upward shift in the DNA band on a native polyacrylamide gel (Fig. 4B) [21, 34]. The addition of all but one null activity mutant, Dut_N^{MgAB}, caused the DNA band to shift back down, indicating a disruption of the Stl-DNA interaction. The addition of Dut_N^{MgAB} instead shifted the band further upward (Fig. 4B), indicating that Dut_N^{MgAB} bound to the Stl-DNA complex without releasing *P_{str}*, perhaps due to a lost ability to bind Mg²⁺ ions.

Release of *P_{str}* is inhibited by dUTP and dUMP

The same EMSA assay was used to test the ability of Dut_{NM1} to cause Stl to release *P_{str}* in the presence of dUTP and dUMP. First, we ascertained that high concentrations (2 mM) of dUTP or dUMP did not interfere with Stl binding to *P_{str}*, which still caused a *P_{str}*-Stl band to appear higher up on the gel (Fig. 5). The WT and mutant Dut_{NM1} proteins were then incubated with Stl, the *P_{str}* DNA, and dUTP or dUMP, and run on a native polyacrylamide gel. In the presence of either dUTP or dUMP, the WT Dut_{NM1} no longer caused the DNA band to shift downward, showing that the ability to bind to Stl and cause the release of *P_{str}* was inhibited by dUTP and dUMP (Fig. 5). The mutants Dut_N^{K159} and Dut_N^{MgAB} also failed to cause the release of Stl from *P_{str}* in the presence of dUTP and dUMP, although Dut_N^{K159} is less inhibited by dUMP than by dUTP. (Note that, as mentioned above, Dut_N^{MgAB} did not release Stl even in the absence of nucleotide at the protein concentration used.) Mutants Dut_N^{FKW}, Dut_N^{MgA} and Dut_N^{MgB} were unaffected in their ability to release cause a downward shift on the gel (Fig. 5). This is most likely due to an impaired ability of these mutants to bind dUTP and dUMP, or perhaps an inability to undergo a nucleotide-dependent conformational change that block the binding of the WT Dut_{NM1} to Stl.

Dut_{NM1} binds to the C-terminal domain of Stl

Nyiri *et al.* [35] showed that the C-terminal domain (CTD) of Stl interacted with the type 1 Dut₁₁. Based on their predictions, we created three N-terminal truncations of Stl: Stl^{48–268} (26 kDa), Stl^{68–268} (24 kDa) and Stl^{84–268} (22 kDa). These truncated Stl proteins were co-expressed with the His₆-tagged WT and mutant Dut_{NM1} proteins. The resulting lysates were passed over a Ni-NTA affinity column, and eluted with increasing concentrations of imidazole. The eluate was evaluated by SDS-PAGE. Full-length Stl (31.5 kDa) was used as a positive control [21]. Both the full-length and truncated Stl proteins co-eluted with WT Dut_{NM1}, indicating that, like Dut₁₁, Dut_{NM1} also interacts with the CTD of Stl (Fig. 6A). A third band observed with Stl^{48–268} could be due to degradation of the flexible linker at the N-terminus of the truncated Stl protein. The affinity purification products were crosslinked with 2% paraformaldehyde. This resulted in the formation of a band that increased in molecular mass with the size of the truncated Stl (Fig. 6B), confirming the interaction

between Dut_{NM1} and the Stl CTD. As expected, the Dut_{NM1} mutants also interacted with Stl^{84–268} (Supplementary Fig. S3).

Dut_{NM1} activity mutants can derepress and mobilize SaPIbov1

To determine whether the mutant Dut_{NM1} proteins with severely impaired dUTPase activity were able to cause mobilization of SaPIbov1, we used allelic exchange to introduce the Dut_N^{FKW} and Dut_N^{MgAB} mutations into the WT ϕ NM1 lysogen RH2, creating mutant phages ϕ NM1 *dut*^{FKW} (ST431) and ϕ NM1 *dut*^{MgAB} (ST438), respectively (Table 3). These mutant phages were then tested for their ability to mobilize SaPIbov1. The WT ϕ NM1 (RH2) and ϕ NM1 *dut* (RH3) lysogen strains were used as controls. All lysogens were induced with mitomycin C to produce phage lysates. Note that since *dut* is not an essential gene, the mutant phages are as viable as the wildtype [21]. The lysates were filtered and used to infect *S. aureus* strains containing either SaPIbov1 *tst::tetM* (JP45) or SaPI1 *tst::tetM* (ST1). If mobilization occurred, the resulting lysates would contain a mixture of phage and *tetM* transducing particles. The transducing titers (TU/ml) were determined by infecting RN4220 and plating in the presence of 5 μ g/ml tetracycline (Fig. 7A). Phage titers (PFU/ml) were determined by plaque assays on *S. aureus* strain RN4220 (Fig. 7B). As expected, WT ϕ NM1 could transduce both SaPIbov1 and SaPI1 (Fig. 7A). ϕ NM1 *dut* failed to transduce SaPIbov1, but retained the ability to transduce SaPI1 (Fig. 7A). Both ϕ NM1 dUTPase null activity mutant phages ϕ NM1 *dut*^{FKW} and ϕ NM1 *dut*^{MgAB} could transduce SaPIbov1 and SaPI1 with titers similar to the wildtype phage (Fig. 7A). These results indicated that dUTPase activity of Dut_{NM1} was not required for the mobilization and transduction of SaPIbov1.

Discussion

80 α and ϕ NM1 are related phages that share a similar genomic organization. Both phages encode a dUTPase in the locus in the replication gene cassette preceding the structural operon [21, 22]. The phage-encoded dUTPases are non-essential for phage growth under experimental conditions, but dUTPases in general are important for genome stability [23, 27, 29]. Strikingly, the enzymes encoded by 80 α and ϕ NM1 represent two structurally distinct classes of dUTPases. Even though the genes are non-homologous, the fact that the dUTPase function is conserved represents a fascinating case of convergent evolution and underscores the importance of these genes.

SaPIs have evolved to take advantage of phage-encoded proteins for their mobilization [19]. We showed previously that the SaPIbov1 Stl repressor could be inactivated by both classes of dUTPases, in spite of their different structures [21, 22]. This led us to speculate that the dUTPase activity itself might be involved in the derepression process. This conjecture was supported by results that dUTP inhibited Stl binding and derepression by the type 1 dUTPases Dut₁₁ and Dut_{80 α} [21, 34, 36]. Furthermore, Stl was found to inhibit the dUTPase activity of both type 1 and type 2 dUTPases [21, 34, 36].

Here, we tested this hypothesis by generating null activity mutants of type 2 Dut_{NM1} and evaluating their ability to bind Stl, release the *P_{str}* promoter and mobilize SaPIbov1 *in vivo*. Our results show that dUTPase activity is not required for either of these functions. All

mutants were able to form heterodimers with Stl, and all mutants except for Dut_N^{MgAB} were able to cause Stl to release *P_{str}* ϕ NM1 lysogens carrying mutant alleles encoding either Dut_N^{FKW} or Dut_N^{MgAB} were able to mobilize SaPI_{bov1} at the same frequency as the WT phage. The ability of Dut_N^{MgAB} to derepress in spite of a failure to release *P_{str}* *in vitro* could be due to differences in the physiological conditions *in vitro* and *in vivo*. Indeed, at high concentrations, Dut_N^{MgAB} is able to cause *P_{str}* release (data not shown).

Only the *apo* form of Dut_{NM1} was effective in causing the release of Stl from *P_{str}*. Stl release by WT Dut_{NM1} was inhibited by both dUTP and dUMP, suggesting that the presence of nucleotide causes a conformational change in the protein that prevents its interaction with Stl (Fig. 8). Some mutants were not inhibited by dUTP and dUMP, either due to an inability to bind the nucleotide or to undergo a nucleotide-dependent conformational change. This is similar to the process in type 1 dUTPases, where dUTP binding and hydrolysis causes conformational changes that alter their ability to bind to Stl [25, 34, 36].

We have also shown that Dut_{NM1}, similar to the type 1 dUTPases [35], interacts with the C-terminal domain of Stl, rather than with the N-terminal DNA binding domain. This demonstrates that the release of the *P_{str}* in both cases is not due to a direct disruption of the Stl-DNA binding interface, but rather is an allosteric effect. Since the active form of Stl is a dimer, the disruption of this dimer through the formation of a Dut_{NM1}-Stl heterodimer provides a mechanism for the *P_{str}* derepression. The type 1 dUTPases, Dut₁₁ and Dut_{80 α} , also work allosterically and probably cause disruption of the Stl dimer, although in this case the Stl binding apparently does not cause a disruption of the dUTPase trimer itself [25, 34, 36]. Upon Stl binding, type 1 dUTPase activity is inhibited due to blocking of the catalytic site [34, 36]. In contrast, inhibition of type 2 dUTPase activity by Stl binding is most likely a coincidental effect of the disruption of the Dut_{NM1} dimer, and not a part of the derepression mechanism *per se* [21].

Even though the structures are different, the possibility remained that the two classes of dUTPases interact with Stl via similar sequence motifs. However, Stl interacting residues identified in Dut_{80 α} and Dut₁₁ [34, 36] are not conserved in Dut_{NM1}. The only conserved sequence between Dut_{80 α} and Dut_{NM1} is the previously noted GVSS motif located at the start of helix α 3 in Dut_{NM1} [21, 22]. This sequence is not conserved in the type 1 dUTPase of phage PH15, which did not mobilize SaPI_{bov1} [19]. However, when we mutated this motif from GVSS to the PH15 sequence (GKSL) in Dut_{NM1}, the protein failed to fold properly (unpublished results). The presence of the GVSS sequence in both classes of dUTPases is most likely a coincidence, and the specific residues in Dut_{NM1} that interact with Stl thus remain to be determined.

Derepression is the first and a critical step in the SaPI lifecycle [10, 12, 19]. It allows for the SaPI to be excised from the genome [30]. If the SaPI fails to be excised when the bacterial cell lyses due to the phage infection, then the SaPI can only be transferred by general transduction, which is at least 1000-fold less efficient than specialized transduction [32, 41]. This would limit the spread of virulence genes to the surrounding cells. A better understanding of the interactions between derepressors and repressors might therefore be important to the development of potential novel phage-based therapies.

Materials and Methods

Cloning, expression and protein purification

Mutations in *dut*_{NM1} were made in the pET21a-based pRH4 plasmid (Table 2) using the Q5 Site-directed mutagenesis kit (New England Biolabs) or QuikChange Lightning kit (Agilent Genomics). Full-length or N-terminally truncated SaPIbov1 *stl* was cloned in tandem with *dut*_{NM1} into the resulting plasmids with its own vector-derived Shine-Dalgarno sequence. The SaPIbov1 Stl CTD was also cloned as a GST fusion into the vector pGEX-6P-1 (GE Healthcare Life Sciences) [21, 34]. The resulting plasmids (Table 2) were verified by DNA sequencing (UAB Heflin Center). All primers were supplied by Eurofins MWG Operon Inc. (Huntsville, AL) and are listed Supplementary Table S1.

The *dut*_{NM1}-containing clones were expressed in *E. coli* BL21 Star (DE3) pLysS (Novagen) and purified as described previously [21, 34], with the following modifications: (1) the temperature was dropped to 32°C after induction; (2) 20µg/ml lysozyme (GoldBioCom) was added to the cell pellet prior to freeze/thaw; and (3) cells were lysed by sonication for 3–6min at 40% output using a Branson Sonifier 250. The lysates were passed over a Ni-NTA affinity column (Bio-Rad), as described previously [21], except for mutant *Dut*_{NM1} co-expressed with truncated Stl, which was purified using a 10 kDa molecular weight cutoff Amicon Pro centrifugal filters (EMD Millipore). GST fusion proteins were expressed in *E. coli* BL21 Star (DE3) pLysS and purified by affinity on glutathione sepharose beads (Amersham Biosciences) [21, 34].

Circular dichroism

CD spectra were acquired on a Jasco J815 spectropolarimeter at 20°C from 260 to 190 nm wavelength, with a continuous scanning rate of 100 nm/min repeated 4 times for each sample. Loading concentrations were 100 µg/ml in a buffer containing 100 mM Na-phosphate (pH 7.8), 200 mM NaF, 1 mM MgCl₂, and 0.15% Tween-20. Each spectrum was smoothed using Savitzky-Golay filtering after subtracting the background signal from the buffer solution. Data were analyzed on the Dichroweb server [42] using reference set 7 [43] and Selecon-3 analysis program [44].

NMR detection of dUTPase activity

dUMP and dUTP (ThermoFisher Scientific) were used as obtained. NMR spectra were recorded at 293K using a Bruker Avance II (700MHz¹H) spectrometer equipped with a cryogenic triple-resonance probe. ¹H chemical shifts of dUMP and dUTP were assigned with the aid of two-dimensional ¹H-¹H COSY, ¹H-¹H TOCSY, and ¹H-¹³C HSQC. ¹H spectrum of free dUTP contained two extraneous doublets at 2.53 and 2.57ppm of an impurity that was identified as citrate. Chemical shifts were referenced to 4,4-dimethyl-4-silapentanesulfonic acid (DSS). dUTPase reactions contained 1 mM dUTP in 100 mM Na-phosphate buffer pH 7.8, 200 mM NaCl, 5 mM MgCl₂, and 5% D₂O. All enzymes were at 200 nM concentration, with the exception of *Dut*_{N^{K159}} and *Dut*_{N^{FKW}}, which were at 500 nM. The progress of dUTP hydrolysis was monitored by recording one-dimensional ¹H spectra with excitation sculpting water suppression. NMR data were collected soon after

addition of the enzyme at defined time intervals. The last data points were collected two weeks after initiation of the reaction.

Pyrophosphate detection assay

dUTPase enzyme activity assays were performed using the EnzCheck Pyrophosphate (PP_i) Assay Kit (Molecular Probes), which measures the release of PP_i upon dUTP hydrolysis, as previously described [21]. 20 μM dUTP was added to 46 nM mutant Dut_{NM1} and the absorbance at 360 nm checked after 30 min at 22 °C in a Bio-Rad SmartSpec spectrophotometer, and compared to a standard curve.

Chemical crosslinking

24 μM WT Dut_{NM1} co-expressed with truncated Stl were crosslinked with 2% paraformaldehyde, as described previously [21]. 3 μM full-length Stl was mixed with 12 μM mutant Dut_{NM1} in EMSA buffer [34] and incubated for 1 hr at 22 °C before crosslinking with 2% paraformaldehyde.

Electrophoretic mobility shift assay

A 57-bp oligonucleotide consisting of the SaPI_{bov1} *P_{str}* promoter region was used in the assay (Supplementary Table S1) [35]. 25–50 ng of *P_{str}* DNA was combined with 1–2 μM Stl both with and without 6–12 μM WT or mutant Dut_{NM1}, and incubated for 30–60 min at 22 °C in EMSA buffer [34]. The mixture was separated on Novex™ 4–20% native Tris-Glycine gels (Life Technologies) [21]. For the nucleotide dependence experiments, 2 mM dUTP or 2 mM dUMP were added to the mixture containing 6–12 μM WT or mutant Dut_{NM1} and 1.5 μM Stl and 25 ng *P_{str}* DNA, and the assay carried out as above.

Allelic exchange

Derivatives of the allelic exchange vector pMAD [45] carrying the *dut*_{NM1}^{FKW} (pLKP18) or *dut*_{NM1}^{MgAB} (pLKP19A) alleles were constructed by assembly of PCR fragments with NcoI digested vector using the In-Fusion® HD Cloning Kit (Clontech Laboratories). The initial *dut*_{NM1}^{MgAB} construct (pLKP19) was missing an adenosine nucleotide in the sequence just 3' of the *dut* coding sequence, due to a discrepancy between the reported sequence (NC_008583), which was used to design primer LKP28, and the sequence that we actually determined for the prophage in RH2; this was corrected in pLKP19A. Plasmids pLKP18 and pLKP19A were introduced into RH2 by electroporation and allelic exchange was performed as described by Arnaud et al [45] to replace the WT *dut* allele on the ϕNM1 prophage with each mutant allele, generating the mutant lysogens ST431 and ST438, respectively (Table 3). All constructs were verified by DNA sequence analysis.

Transduction assay

Phages were prepared by mitomycin C induction of the lysogens listed in Table 3 [21]. Cultures of SaPI-containing strains (ST1 and JP45) were grown in CY+GL [46] at 32 °C in the presence of 5 μg/ml tetracycline. At A₆₀₀ = 0.6, the cells were diluted with phage buffer and infected with phage at an approximate m.o.i. = 1. The resulting lysates were collected

after 4hrs and filtered. Phage titer and SaPI transduction assays were performed as described previously [21].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *S. aureus* pathogenicity islands (SaPIs) are mobilized by helper phages
- SaPI_{bov1} StI is derepressed by the type 2 dUTPase of bacteriophage ϕ NM1 (Dut_{NM1})
- Mobilization of SaPI_{bov1} by ϕ NM1 does not require Dut_{NM1} dUTPase activity
- Derepression of StI by Dut_{NM1} is inhibited by dUTP and dUMP

Figure 1.

Mutation of active site residues in Dut_{NM1}. (A) Threading sequence alignment of Dut_{NM1} (fNM1_Dut) with *C. jejuni* dUTPase (Cjej_Dut) generated with I-TASSER [21, 47] and displayed using ESPrict 3.0. Locations of α-helices in the Dut_{NM1} model are shown below the alignment. Locations of active site mutations are indicated: Dut_N^{MgB}, blue circles; Dut_N^{FKW}, red triangles; Dut_N^{MgA}, purple circles; Dut_N^{K159}, green square; Dut_N^{MgAB}, all circles. (B) Ribbon representation of the I-TASSER prediction of Dut_{NM1}. The left panel shows one active site, comprised of residues from both subunits (yellow and gray), which are indicated in stick model, colored and labeled according to (A). The right panel shows the whole dimer with both active sites indicated in stick model.

Figure 2. Mutant Dut_{NM1} CD spectra and crosslinking. (A) CD spectra of purified WT and mutant Dut_{NM1} -His₆ proteins at 100 $\mu\text{g}/\text{ml}$ concentration, colored as indicated. (B) Coomassie stained 4–15% SDS-PAGE of purified WT and mutant Dut_{NM1} either untreated (–) or crosslinked with 2% paraformaldehyde for 30 min (+). Bands corresponding to Dut monomers (arrowhead) and Dut dimers (arrow) are indicated. M, marker, molecular weights (kDa) indicated.

Figure 3.

Progress of dUTP hydrolysis of WT and mutant Dut_{NM1} detected by ¹H-NMR. For each spectral overlay, the black spectrum is for free dUTP, while the red, blue, and green spectra were obtained at the indicated time points following enzyme addition. ¹H signal assignments for dUTP (black) and dUMP (blue) are indicated at the bottom. Asterisks indicate signals of residual imidazole.

Figure 4.

Stl interaction with mutant Dut_{NM1} . (A) Coomassie stained 4–15% SDS-PAGE of WT and mutant Dut_{NM1} with Stl, untreated (–) or crosslinked with 2% paraformaldehyde for 30 min (+). Bands corresponding to Dut_{NM1} and Stl monomers (arrowheads), Dut_{NM1} dimers (Dut_2) and the Dut : Stl heterodimer (arrows) are indicated. M, marker, molecular weights in kDa. (B) Electrophoretic mobility shift assay. A 57-bp DNA oligomer corresponding to the P_{str} promoter region of SaPIbov1 was incubated with Stl and/or WT or mutant Dut_{NM1} and separated on a native 4–20% polyacrylamide gel, followed by ethidium bromide staining.

The gel image contrast was inverted for clarity. Bands corresponding to unbound P_{str} DNA (arrowhead) and the Stl-DNA complex (arrow) are indicated. The asterisk shows a band apparently corresponding to a Dut_N^{MgAB} -Stl-DNA complex.

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Figure 5.

Electrophoretic mobility shift assay showing the effect of nucleotide. SaPIbov1 P_{str} DNA was incubated with Stl and WT or mutant Dut_{NM1} in the presence of 2 mM dUTP (A) or dUMP (B) and analyzed as in Fig 4. Bands corresponding to the 57-bp P_{str} DNA and the DNA-Stl complex are indicated.

Figure 6.

Interaction of Stl CTD and Dut_{NM1}. (A) Coomassie-stained 4–15% SDS-PAGE of the nickel affinity pulldown of WT Dut_{NM1}-His₆ (arrow) co-expressed with full length (FL) and N-terminally truncated Stl (arrowheads). (B) Coomassie stained 4–15% SDS-PAGE of co-purified Dut_{NM1}-His₆ and FL or N-terminally truncated Stl, untreated (–) or crosslinked with 2% paraformaldehyde (+). Bands corresponding to Dut_{NM1} (arrow) and the Dut_{NM1}–Stl heterodimers (arrowheads) are indicated. M, marker, molecular weights (kDa) indicated.

Figure 7.

Transduction assay. *S. aureus* strains containing SaPI1 *tst::tetM* (ST1; green) and SaPIbov1 *tst::tetM* (JP45; purple) (Table 3) were infected with WT ϕ NM1, ϕ NM1 Δ dut, ϕ NM1 dut^{FKW} or ϕ NM1 dut^{MgAB} , as indicated. In (A), the resulting lysates were used to infect RN4220 and plated on GL agar in the presence of tetracycline for calculation of transducing titers (TU/ml). (B) Phage titers (PFU/ml) of the resulting lysates, assayed by plating on RN4220 in phage agar. The bars represent the average of three experiments; standard deviations are indicated.

Figure 8.

Schematic diagram showing the derepression process. Only the *apo* form of *Dut*_{NM1} is able to bind the C-terminal domain of Stl and disrupt the Stl dimer, causing release of the *P*_{str} promoter. The presence of dUTP or dUMP prevents the Stl interaction.

Table 1Characteristics of ϕ NM1 Dut mutations

Name	Mutation	Location	Potential Mechanism	Functionally Active	Dimer	% α -helical	
						Selecon 3	NRMSD
WT DutNM1	–	–	–	Y	Y	44%	0.082
Dut _N ^{K159}	K159A	α 8	Phosphate binding	N	Y	26%	0.090
Dut _N ^{FKW}	F50A K51A W53A	α 2– α 3 loop	Uracil binding and pocket formation	N	Y	53%	0.066
Dut _N ^{M6A}	E67Q D70N	α 3	Mg ²⁺ binding site	N	Y	37%	0.099
Dut _N ^{M6B}	E39Q E42Q	α 2	Mg ²⁺ binding site	N	Y	10%	0.165
Dut _N ^{M6AB}	E39Q E42Q E67Q D70N	α 2, α 3	Both Mg ²⁺ binding sites	N	Y	14%	0.257

NRMSD is a fit parameter, representing the RMS difference between the experimental ellipticities and the ellipticities of the back-calculated spectra. Lower values have better fit [48].

Table 2

List of plasmids used in this study.

Plasmid	Description	Reference/Source
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 vector	[45]
pLKP18	pMAD derivative with <i>dut</i> _{NM1} ^{FKW}	This study
pLKP19	pMAD derivative with <i>dut</i> _{NM1} ^{MgAB}	This study
pLKP19A	pMAD derivative with <i>dut</i> _{NM1} ^{MgAB} + extra intergenic A	This study
pRH4	pET21a <i>dut</i> _{NM1} -His ₆	[21]
pRH5	pET21a <i>dut</i> _{NM1} -His ₆ , <i>stl</i>	[21]
pRH24	pGEX-6P <i>stl</i>	[21]
pRH25	pET21a <i>dut</i> _{NM1} -His ₆ K159A	This study
pRH26	pET21a <i>dut</i> _{NM1} -His ₆ F50A K51A W53A	This study
pRH27	pET21a <i>dut</i> _{NM1} -His ₆ E67Q D70N	This study
pRH28	pGEX-6P <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH29	pGEX-6P <i>stl</i> ⁶⁸⁻²⁶⁸	This study
pRH30	pGEX-6P <i>stl</i> ⁴⁸⁻²⁶⁸	This study
pRH31	pET21a <i>dut</i> _{NM1} -His ₆ , <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH32	pET21a <i>dut</i> _{NM1} -His ₆ , <i>stl</i> ⁶⁸⁻²⁶⁸	This study
pRH33	pET21a <i>dut</i> _{NM1} -His ₆ , <i>stl</i> ⁴⁸⁻²⁶⁸	This study
pRH34	pGEX-6P <i>stl</i> Q40A N41A	This study
pRH35	pET21a <i>dut</i> _{NM1} -His ₆ E39Q E42Q	This study
pRH36	pET21a <i>dut</i> _{NM1} -His ₆ E39Q E42Q E67Q D70N	This study
pRH37	pET21a <i>dut</i> _{NM1} -His ₆ K159A, <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH42	pET21a <i>dut</i> _{NM1} -His ₆ E67Q D70N, <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH45	pET21a <i>dut</i> _{NM1} -His ₆ F50A K51A W53A, <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH48	pET21a <i>dut</i> _{NM1} -His ₆ E39Q E42Q, <i>stl</i> ⁸⁴⁻²⁶⁸	This study
pRH51	pET21a <i>dut</i> _{NM1} -His ₆ E39Q E42Q E67Q D70N, <i>stl</i> ⁸⁴⁻²⁶⁸	This study

Table 3

List of bacterial strains used in this study.

Description		Reference/Source
<i>E. coli</i> strains:		
BL21 Star (DE3) pLysS	Expression strain, increased RNA stability	Novagen
DH5 α	Recombination-deficient cloning strain	Novagen
<i>S. aureus</i> strains:		
RN4220	RN450, mutated to accept foreign DNA	[49]
JP45	RN4220 (<i>SaPI</i> bov1 <i>tst::tetM</i>)	[50]
RH2	RN4220 (ϕ NM1)	[21]
RH3	RN4220 (ϕ NM1 <i>dut</i>)	[21]
ST1	RN4220 (SaPI1 <i>tst::tetM</i>)	[50]
ST431	RN4220 (ϕ NM1 <i>dut</i> ^{FKW})	This study
ST438	RN4220 (ϕ NM1 <i>dut</i> ^{MgAB})	This study