Processing of Non-Conjugative Resistance Plasmids by Conjugation Nicking Enzyme of Staphylococci

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ABSTRACT

Antimicrobial resistance in Staphylococcus aureus presents an increasing threat to human health. This resistance is often encoded on mobile plasmids such as pSK41; however, the mechanism of transfer of these plasmids is not well understood. First, we examine key protein-DNA interactions formed by the relaxase enzyme, NES, which initiates and terminates the transfer of multidrug resistance plasmid pSK41. Two loops on the NES protein, Hairpin Loop 1 and 2, form extensive contacts with the DNA hairpin formed at the oriT of pSK41, and here we establish that these contacts are essential for proper DNA cleavage and religation by the full 665-residue NES protein in vitro. Second, pSK156 and pCA347 are non-conjugative Staphylococcus aureus plasmids that contain sequences similar to the oriT of pSK41 but differ in the sequence predicted to form a DNA hairpin. We show that pSK41 NES is able to bind, cleave, and religate the oriT sequences of these non-conjugative plasmids in vitro. Although pSK41 could mobilize a co-resident plasmid harboring its cognate oriT, it was unable to mobilize plasmids containing the pSK156 and pCA347 variant oriT mimics, suggesting that an accessory protein like that previously shown to confer specificity in the pWBG749 system may also be involved in transmission of plasmids containing a pSK41-like oriT. These data indicate that the conjugative relaxase-in trans mechanism recently described for the pWBG749 family of plasmids also applies to the pSK41 family of plasmids, further heightening the potential significance of this mechanism in the horizontal transfer of staphylococcal plasmids.
IMPORTANCE

Understanding the mechanism of antimicrobial resistance transfer in bacteria such as Staphylococcus aureus is an important step toward potentially slowing the spread of antimicrobial resistant infections. This work establishes protein-DNA interactions essential for the transfer of the Staphylococcus aureus multiresistance plasmid pSK41 by its relaxase NES. This enzyme also processed variant oriT-like sequences found on numerous plasmids previously considered non-transmissible, suggesting that in conjunction with an uncharacterized accessory protein, they may be capable of transferring horizontally via a relaxase-in trans mechanism. The findings have important implications for our understanding of staphylococcal resistance plasmid evolution.

INTRODUCTION

Antimicrobial resistant strains of Staphylococcus aureus are a growing concern for hospital- and community-acquired infections. Most S. aureus bacteria examined clinically harbor at least one plasmid that encodes for antimicrobial resistance, and many plasmids carry multiple antimicrobial resistance determinants. The pSK41 family of plasmids is made up of large, low-copy-number, conjugative plasmids for which pSK41 is used as a prototype for characterization (1-4). These plasmids carry a variety of antimicrobial resistance determinants, including those against aminoglycosides, penicillins, tetracycline, bleomycin, trimethoprim, macrolides, lincosamides, mupirocin, antiseptics, and disinfectants (2, 4-9). This family of plasmids also played a key role in the rise of vancomycin resistant S. aureus (VRSA) (7, 8, 10). In addition to the antimicrobial resistance, they also carry transfer (tra) genes encoding the proteins necessary to conduct conjugative plasmid transfer that
spread these plasmids among *S. aureus* and other gram-positive bacteria (6, 7, 10, 11).

One of the proteins essential for conjugative plasmid transfer is the relaxase enzyme. A relaxase is responsible for initiation and completion of the transfer process as it cleaves one strand of the double stranded plasmid to begin transfer, and then ligates that strand back together to complete transfer (8, 12-15). There are two classes of relaxases: multi-tyrosine relaxases that use a “thumb” motif to position the plasmid DNA for processing, and single-tyrosine relaxases which lack this thumb motif (9, 16). The relaxase of pSK41 is termed NES, nicking enzyme in *S. aureus*, and is a single-tyrosine relaxase (1-6, 8, 9). NES contains a relaxase N-terminal 220 residues and a C-terminal 350 residues necessary for *in vivo* function but via an uncertain mechanism (5, 7, 8, 10). The crystal structure of the relaxase domain of NES was the first of a single-tyrosine relaxase bound to its target DNA, allowing for more detailed characterization of the protein-DNA interactions than previously possible (6-8, 11). This structure revealed two sets of important protein-DNA interactions. The first is that the “thumb” used by multi-tyrosine relaxases to position the DNA appears to be replaced by 12 protein-DNA contacts including a buried nucleotide 3 bases upstream of the *nic* site that places the DNA in the correct position to be nicked by the single, catalytically active tyrosine. The second set of protein-DNA interactions unique to the NES-DNA complex is composed of two protein loops, termed Hairpin Loop 1 and 2, that surround the DNA hairpin formed upstream of the *nic* site (Figure 1A and B). NES Hairpin Loop 1, shown in yellow in Figure 1A and B, forms two base specific contacts with the minor groove of the oriT DNA and one contact to the phosphate backbone. NES Hairpin Loop 2, green in
Figure 1A and B, contacts the DNA more extensively, with six base specific interactions and four phosphate contacts in the major groove of the DNA hairpin. Edwards et al. previously showed in vitro that these loops disrupt DNA cleavage by the relaxase domain alone and in vivo that full-length NES protein lacking these loops was not able to facilitate plasmid transfer (7, 8, 10, 14). However, this important protein-DNA interaction had not been characterized in vitro in the context of the full-length 665-residue NES protein and we set out to determine in which steps of conjugation this interaction plays a role.

pSK41-like conjugative plasmids have also been shown to mobilize several smaller co-resident plasmids such as pC221 and pSK639, which encode their own mob genes (9, 12, 13, 15, 16). Recently, O’Brien et al. showed that another staphylococcal conjugative plasmid pWBG749, which is unrelated to pSK41, can facilitate the mobilization of other plasmids that lack mob genes (17). They demonstrated that this transfer is facilitated by origin-of-transfer sequences on the mobilizable plasmids that mimic the pWBG749 origin-of-transfer sequence, suggesting a conjugative relaxase-in trans mechanism (18). We have identified sequences similar to the pSK41 origin of transfer on numerous non-conjugative staphylococcal resistance plasmids (Supplemental Datasets S1 and S2), raising the possibility that pSK41 family plasmids might likewise facilitate mobilization of other plasmids by an analogous relaxase-in trans mechanism mediated by NES. To investigate this possibility, we have characterized the pSK41 oriT mimic sequences from two divergent non-conjugative plasmids. The first plasmid, pSK156, was isolated from a clinical strain in 1951 and is the earliest known multidrug efflux-encoding plasmid (19). The second plasmid, pCA347, was first sequenced in 2013.
after isolation from a USA600 methicillin-resistant strain of S. aureus and encodes resistance to penicillin and heavy metals (20). Importantly, the variation in the origin-of-transfer sequence of pSK41 and the mimics of pSK156 and pCA347 is in the hairpin region of the DNA (see Figure 3A). Based on these observations we sought to explore the ability of NES to bind to and process putative oriT regions from pSK156 and pCA347 and the ability of pSK41 to facilitate transfer of plasmids containing these putative oriT regions in order to examine the potential for mobilization of plasmids containing pSK41 oriT mimics in staphylococci.

MATERIALS AND METHODS

Cloning, Expression and Purification of NES. Wild-type full-length NES was previously cloned into the cysteine protease domain (CPD) fusion protein expression system developed by Shen et al. and optimized by this lab (7, 21). Loop deletion mutants were made through site directed mutagenesis to remove Hairpin Loop 1 (residues 77 to 82) and Hairpin Loop 2 (residues 150 to 157) and replace each with a linker composed of one glycine and one serine. Cleavage inactive mutants used in binding studies were made by replacing the tyrosine at amino acid position 25 with a phenylalanine. The resulting plasmids were transformed into Escherichia coli BL21 (DE3) Al cells and grown in 1.5L of lysogeny broth (LB) in the presence of 0.1 mg/ml ampicillin at 37°C with shaking. At an optical density of 0.6-0.8, an L-Arabinose solution was added at a final concentration of 0.2% (vol/vol) and the temperature reduced to 18°C. After 30 minutes, protein expression was induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG) and cells were allowed to grow for 16 hours. The cells were pelleted and stored at -80°C. Individual cell pellets were resuspended in buffer A [500 mM NaCl, 20 mM KH₂PO₄ pH 7.4, 25 mM Imidazole,
0.02% (v/v) sodium azide] along with protease inhibitor tablets (Roche), DNase, and lysozyme. The mixture was sonicated and then clarified via centrifugation. The supernatant was filtered and loaded onto a HisTrap column (GE Healthcare). The CPD expression system contains a His6 tag in addition to the CPD tag, which has self-cleavage abilities in the presence of inositol hexakisphosphate (InsP6). Therefore, after the His-CPD-NES fusion protein was bound to the column via the His6 tag, the column was incubated with 2 mM InsP6 for 3 hours at 4°C. The NES protein was then eluted off the column in buffer A while the His6 and CPD tags remained bound to the column. The NES protein was then passed over a Superdex 200 column (GE Healthcare) pre-equilibrated in sizing buffer [25 mM HEPES pH 7.4, 300 mM NaCl, 0.02% (v/v) sodium azide]. Purity of each fraction was assessed by SDS-PAGE gel and fractions containing >95% pure protein were combined and concentrated to approximately 1.2 mg/ml.

DNA Binding Studies. 5'-end 6-FAM labeled DNA oligos were ordered from Integrated DNA Technologies, resuspended in annealing buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.05 mM EDTA), and hairpin was formed by heating the oligo to 98°C for one minute and then cooling the solution by 3°C per second. The dissociation constant of binding was calculated using fluorescence anisotropy as described in Edwards et al. (7). Briefly, protein was serially diluted into a buffer of 100 mM NaCl, 0.1 mg/ml BSA, 5 mM Magnesium Acetate, 25 mM Tris Acetate, pH 7.5 to give 40 μL at final protein concentrations ranging from 0 to 0.5 μM. Assays were conducted in a 384-well black assay plate (Costar) allowing for 16 concentrations of protein. 10 μL of the DNA probe was added to the 40 μL protein solution resulting in a final concentration of DNA of 50 nM in a total volume of 50 μL in each well. Fluorescence
anisotropy of the fluorescein-labeled DNA was observed via excitation at 485 nm and emission at 520 nm using a PHERAstar plate reader (BMG Labtech).

Measurements were made in triplicate and reported values are the average of three separate triplicate runs. Data were plotted as average fluorescence anisotropy as a function of protein concentration using Graphpad PRISM v6.05 (Graphpad, 2014).

The following equation was employed to fit the data and to calculate the $K_D$ for the substrate:

$$f = \min + \frac{(\max - \min)(T + x + K) - \left\{(-T - x - K)^2 - 4Tx\right\}^{\frac{1}{2}}}{2T}$$

where $f$ is average fluorescence anisotropy signal; $T$, total DNA concentration (set to 50 nM); $x$, total protein concentration; $K$, $K_D$; $\min$, average fluorescence anisotropy signal of no protein control; and $\max$, average fluorescence anisotropy signal of sample at saturating concentration of protein. A single binding site was assumed and standard error is reported for each measurement. The reported values are an average of at least 5 independent experiments.

**DNA Cleavage Assays.** The same 5′-end 6-FAM labeled DNA oligos used for the DNA binding studies were used to measure equilibrium DNA cleavage via polyacrylamide gel electrophoresis (PAGE) gels. Each 10 μL reaction contained 1.52 μM NES protein, 1 μM DNA substrate, and EMSA buffer (50 mM NaCl, 20 mM Tris, pH 7.4, 0.02% (vol/vol) sodium azide). The reaction was incubated at 37°C for 1 hour and quenched by the addition of 2X running buffer (0.01% xylene cyanol, 0.01% Bromophenol Blue, 85% formamide, 20 mM EDTA, 2X TAE, 0.2% SDS). The resulting 20 μL reactions were run through a denaturing 16% polyacrylamide gel [35 mL 16% acrylamide gel stock (8 M urea, 16% polyacrylamide/bisacrylamide, 1X]
TBE), 300 μL 10% ammonium persulfate (APS), 33 μL tetramethylethylenediamine (TEMED) in 1X TBE running buffer to separate cleaved product DNA from the substrate. Using the fluorescein tag, oligos were visualized using a VersaDoc Imaging System, 4400 MP (BioRad) and the QuantityOne software (BioRad). ImageJ 1.45s software was used to quantify band intensities and the percent cleavage product formation was calculated as a percentage of the product band intensity divided by the product plus the substrate band intensities. The average of at least six individual cleavage experiments are presented, individual data points are available in Figures S2, S3, and S5.

**DNA Strand Transfer Assays.** DNA strand transfer assays were performed similarly to DNA cleavage assays except two pieces of DNA were used. The first piece of DNA was an unlabeled substrate of the same sequence and length as the oligos used in the DNA binding and cleavage studies (red DNA in Figure 2B). The second piece of DNA was a 5'-end 6-FAM labeled DNA oligo of the same sequence as the unlabeled substrate, but ending at the NES cleavage site (black DNA in Figure 2B). Each 10 μL reaction contained 1.52 μM NES protein, 1 μM unlabeled DNA substrate, 1 μM labeled DNA substrate, and EMSA buffer. The reaction was incubated, run, and analyzed as in the DNA cleavage assays. Percent strand transfer was calculated as a percentage of the product band intensity divided by the product plus the labeled substrate band intensities. The unlabeled DNA substrate was not visualized or quantified. The average of at least six individual cleavage experiments are presented, individual data points are available in Figures S2, S4, and S5.
**Structure Modeling.** The NES relaxase domain-DNA complex structure reported previously (Edwards et al.; RCSB accession code 4HT4) was employed for Figures 1A-B and 3B-C. (7). For Figures 3D and E in which pSK156 and pCA347 were modeled in place of the original pSK41 DNA, Coot was used to mutate each DNA residue, and the final figures were rendered in PyMol (22, 23).

**Plasmid Sequence Analysis.** The plasmid database compiled and analyzed for pWBG749 family oriT sequences by O’Brien et al. was analyzed for oriT sequences similar to that of pSK41 (18). The online interface of BLASTN was used to search these plasmids for the sequence ATAAGTGCGCCCTTACGGGATTTAAC from the pSK41 oriT and each sequence with a match was manually inspected for an adjacent DNA hairpin sequence (24). Plasmids were then grouped according to varying sequences found in the DNA hairpin. Plasmids determined to carry potential pSK41 oriT- mimics were then searched for the NES relaxase gene (Accession Code: NC_005024.1, nucleotides 8115 to 10112) to determine if the plasmid is a conjugative plasmid.

**Bacterial strains, plasmids, growth and assay conditions.** Strains and plasmids used are listed in Supplementary Table S1. E. coli and S. aureus were cultured at 37°C on LB agar or in liquid LB medium with aeration (200 rpm). When required, growth medium was supplemented with antibiotics at the following concentrations: ampicillin (Ap) 100 μg/mL; chloramphenicol (Cm) 10 μg/mL; gentamicin (Gm) 20 μg/mL; novobiocin (Nb) 5 μg/mL; streptomycin (Sm) 50 μg/mL.
DNA fragments encompassing oriT regions were synthesised as GeneArt Strings (Supplementary Figure S6; Life Technologies) and cloned into HindIII and/or BamHI sites of the pSK1-based S. aureus/E. coli shuttle vector pSK5632. The insert integrity was verified by sequencing. pSK5632 constructs were introduced into the restriction-deficient S. aureus strain RN4220 by electroporation. pSK41 was introduced into each resulting strain by conjugation with strain SK5428 and resulting Cm<sup>R</sup>/Gm<sup>R</sup>-transconjugants were used as donors in mobilization experiments. Mobilization assays were conducted in BHI liquid medium (Sigma Aldrich) containing 40% (final) polyethylene glycol (PEG) as described previously (O'Brien et al., 2015). The WBG4515 strain was used as a recipient and Sm/Nb was used to select against donors. Transconjugants were isolated on media additionally carrying either Gm (for pSK41) or Cm (for pSK5632).

RESULTS

Characterization of NES Hairpin Loop 1 and 2

The crystal structure of the relaxase domain of NES in complex with pSK41 oriT DNA hairpin, reported previously by Edwards et al., revealed two features unique to this class of relaxase: two protein loops, termed Hairpin Loop 1 and 2 (Figure 1A and B), that clamp around the hairpin duplex of the oriT DNA (7). These contacts are unique to this class of relaxase compared to those observed with the longer, multi-tyrosine relaxases like F TraI, and they have yet to be characterized in the context of the full-length protein. Hence, we sought to determine the impact deleting these unique loops would have on NES functions in vitro. Hairpin Loop 1 deletion (ΔL1), Hairpin Loop 2 deletion (ΔL2), or double-deletion (ΔL1ΔL2) forms of full-length NES protein were created using site-directed mutagenesis in which the loops were
replaced with Gly-Ser linkers. The proteins were expressed recombinantly in *E. coli* and purified to homogeneity. DNA binding, cleavage, and strand transfer assays were conducted using DNA oligonucleotides similar to that employed in the complex presented in the crystal structure and possessing the same sequence as the origin of transfer (*oriT*) of NES conjugated plasmid pSK41 (Figure 1C).

For DNA binding studies, these variant proteins contained an active site Tyr-25-Phe mutation. Previous evidence suggested NES relaxase activity is dependent on oligonucleotide length (Figure S1, S2, 7) so varying lengths of the *oriT* were used to verify this trend and named as in Figure 1C. Longer oligonucleotides should better mimic *in vivo* plasmid transfer. As shown in Figure 2A, the ΔL1 form of full-length NES exhibited increased DNA binding (p<0.005) compared to wild-type NES on the OriTHP35 and OriTHP40 oligonucleotides. ΔL2 NES did not demonstrate significantly different DNA binding on any oligo. In contrast, ΔL1ΔL2 NES showed significantly increased DNA binding (p=0.0002) on the shortest oligo tested, OriTHP30, but decreased binding (p<0.005) on OriTHP35. For the longest oligonucleotide tested, OriTHP45, no difference in binding was observed for any variant proteins compared to wild-type NES. Thus, we conclude that eliminating Hairpin Loops 1 or 2 from full-length NES can alter DNA binding *in vitro* in an oligonucleotide length-dependent manner.

DNA cleavage and strand transfer assays were conducted as described in Figure 2B. The cleavage assay mimics the cleavage of the plasmid *oriT* to produce the single strand transferred during conjugation. On OriTHP30, only ΔL1 NES exhibited a significant (p<0.0001) difference in DNA cleavage, in this case a reduction, relative
to wild-type NES (Figure 2C). On the longer OriTHP35, OriTHP40 and OriTHP45 oligos, all three variant proteins (ΔL1, ΔL2, ΔL1ΔL2) demonstrated statistically significant (p<0.0001) increases in levels of DNA cleavage relative to wild-type NES. For these longer oligos, wild-type cleavage was observed at ~4%, while the variant proteins exhibited 2- to 7-fold increases in cleavage. We conclude that eliminating the DNA hairpin-associating loops from NES increases DNA cleavage by the enzyme.

A more dramatic effect was observed in examining DNA strand transfer by the variant full-length forms of NES. The strand transfer assay measured the ligation of a portion of DNA covalently linked to NES following cleavage to a new piece of DNA containing the hairpin characteristic of the oriT (Figure 2B). This mimics the ligation step of conjugation that ends plasmid transfer. For OriTHP30, all NES variants (ΔL1, ΔL2, and ΔL1ΔL2) showed 5- to 15-fold increases in DNA strand transfer relative to wild-type NES (Figure 2D). For OriTHPs 35, 40 and 45, the increases were even larger – 25% to nearly 50% of the substrate oligos provided to the NES variants were processed to strand transfer, while ~5% of the oligos formed strand transfer products with wild-type NES. Thus, eliminating the DNA hairpin contacting loops of NES produces significant and dramatic increases in the level of DNA strand transfer in vitro compared to wild-type NES. It can be concluded that the Hairpin Loop 1 and Loop 2 regions of NES play an important role, particularly on longer DNA substrates more relevant to transfer in vivo, in limiting the level of DNA religation during conjugation.

Modeling of NES Bound to pSK156 and pCA347
We next sought to determine if related DNA sequences from other plasmids might serve as substrates for pSK41 NES. We examined the *S. aureus* plasmids of known sequence and selected two with sequences identical to the pSK41 ori*T cleavage site. These two plasmids, pSK156 and pCA347, exhibited the same sequence as pSK41 in the 20 nucleotide region from the predicted hairpin through the nic site, but deviated somewhat from the DNA hairpin region of the pSK41 ori*T (Figure 3A). We predicted based on modeling that pSK156 and pCA347 might each form 8 base pair DNA hairpins with a one-nucleotide bulge; by contrast, pSK41 is known from crystal structure to form a 7 base pair DNA hairpin with no bulge (Figure 3A and B).

Interestingly, within the predicted DNA hairpins of pSK156 and pCA347, nucleotides at the base of the DNA hairpin (G₃, C₁₇ and G₁₈) are conserved with the sequence of pSK41 (Figure 3A, C, D and E). Furthermore, we noted that the 8 base pair hairpins predicted for pSK156 and pCA347 are nearly identical in sequence to each other (Figure 3A, D and E).

We next modeled the pSK156 and pCA347 DNA sequences into the pSK41 NES relaxase domain-DNA hairpin complex crystal structure. For reference, Figure 3B shows the NES relaxase domain in complex with the pSK41 DNA hairpin, highlighting the interactions between the protein and DNA; the boxed region contains all the protein contacts with the DNA hairpin and will remain the focus of the pSK156 and pCA347 models. As shown in Figure 3C, NES makes base specific contacts with the pSK41 DNA hairpin at C₄ via N154, T₁₆ via R78, C₁₇ via G153 and N154, G₁₈ via R151, and C₁₉ via Y156. All but one contact to pSK156 and two contacts to pCA347, along with six phosphate contacts, are maintained in the models despite the changes in the DNA sequences between these plasmids and pSK41 (Figures 3D
and E). Because C₁₇ and G₁₈ are conserved in both pSK156 and pCA347, the contacts via G₁₅₃, N₁₅₄, and R₁₅₁ are maintained. The cytosine at position 19 in pSK41 is replaced by a thymine in pSK156 and pCA347; however, the para-oxygen of thymine appears capable of receiving a hydrogen bond from Y₁₅₆ of NES. Position 16 of pSK156 and pCA347 contains an adenine rather than the thymine found in pSK41. The ring nitrogen of adenine appears capable in our models of receiving the same hydrogen bond from R₇₈ as the oxygen of thymine; however, while the thymine oxygen can form two hydrogen bonds, the adenine nitrogen can form only one. Thus, in spite of sequence differences between pSK41 and these other two S. aureus plasmids, contacts between NES and the predicted oriTs of all three plasmids are largely maintained.

An additional contact is predicted to be lost between NES and pCA347. While a cytosine is conserved in the same positions in pSK41 (position -4) and pSK156 (position -2), it is a thymine in pCA347 (T₂; Figure 3E). In pSK41 and pSK156 the amine group of C₄ donates a hydrogen bond to the oxygen of N₁₅₄; however, the para-oxygen of thymine cannot form the same interaction. However, it is possible that the asparagine side chain could rotate to allow the thymine oxygen to receive a hydrogen bond from the N₁₅₄ side chain amine. In doing so, though, this side chain would lose an interaction with C₁₇. Despite this potential change, five base-specific contacts and six phosphate contacts are maintained in our models between NES and the sequences of plasmids pSK156 and pCA347 in this region. Thus, we hypothesize that NES is capable of binding to and utilizing these potential oriT regions of pSK156 and pCA347 as substrates.
Characterization of NES Processing of pSK156 and pCA347

We next analyzed the ability of pSK41 NES to process the potential oriT regions of pSK156 and pCA347 by measuring the protein's ability to employ these DNAs for binding, cleavage and strand transfer. For DNA binding studies, wild-type full-length NES with an active site Y25F mutation was employed along with OriTHP40-like forms of the pSK41, pSK156, and pCA347 (Figure 3A). The OriTHP40-like form alone was analyzed as longer oligonucleotides have been shown to be important for the regulatory function of the C-terminal domain (Figure S1, S2, 7) but significant differences between OriTHP40 and 45 were not seen in assays with the NES loop deletion protein mutants. NES bound the oriT mimic regions of pSK156 and pCA347 but less well compared to its binding of the pSK41 oriT (Figure 4A). The K_D of NES binding to pSK41 is 19.3 ± 3 nM; in contrast, NES binds to pSK156 and pCA347 3- and 9-fold weaker, with K_D's of 55.8 ± 9 nM and 175 ± 30 nM, respectively. While the loss of one or two hydrogen bonds is not sufficient to explain this decrease in binding affinity, it is interesting that the changes in binding affinity reflect the degree of change in sequence and interactions seen in our models.

DNA cleavage and strand transfer assays with full-length wild-type pSK41 NES and the OriTHP40-like regions of pSK156 and pCA347 were conducted as described in Figure 2B. For DNA cleavage, pSK156 exhibited the same level of activity as pSK41, while pCA347 showed significantly decreased cleavage by NES (p<0.0001; Figure 4B). However, as the cleavage process is dependent on NES first binding the DNA, this reduction in cleavage may result from the decrease in binding seen in Figure 4A for pCA347. For DNA strand transfer, both pSK156 and pCA347 showed significantly increased DNA strand transfer, with 3- and 7-fold increases in strand...
transfer for pSK156 and pCA347, respectively, relative to pSK41 (Figure 4C). Taken together, these DNA binding, cleavage, and strand transfer data reveal that NES is capable of processing pSK156 and pCA347 oriT-like sites but does so at lower efficiency than at its cognate site.

Relaxase-in trans Mobilization by pSK41 In Vivo

To investigate the ability of pSK41 to facilitate relaxase-in trans mobilization, the oriT-like sites corresponding to pSK156 and pCA347, and the pSK41 oriT sequence itself were synthesized and cloned into the non-mobilizable shuttle vector pSK5632 (25) to generate the plasmids pSK6881, pSK6879 and pSK6877, respectively; the DNA fragments cloned are shown in Figure S6. These new plasmid constructs and pSK5632 were electroporated into S. aureus strain RN4220 and pSK41 was subsequently introduced via conjugation. These strains were then used as donors in mobilization assays with the recipient strain S. aureus WBG4515. As shown in Table 1, pSK41 was found to mobilize pSK6877, containing its own cognate oriT sequence, at a frequency of 2.9 x 10^{-5}, approximately five-fold lower than pSK41 itself transferred in the same assay (1.4 x 10^{-4}). However, despite repeated efforts, mobilization of the plasmids containing the pSK156 or pCA347 oriT mimics was never detected. These results demonstrate that pSK41-encoded NES can mediate in trans mobilization of a plasmid containing a copy of its own oriT site, but suggest its activity on the variant oriT-like sites from pSK156 and pCA347 is inadequate to facilitate plasmid transfer in vivo. As discussed below, an accessory protein may be required to complete relaxase-in trans transfer in vivo.

DISCUSSION
Transfer of conjugative and mobilizable plasmids is a major route by which antimicrobial resistance propagates, but the lack of details about the mechanism of this process impedes efforts to slow or prevent the spread of such resistance. We focus on the mechanism of action of the NES relaxase enzyme encoded by pSK41 and related plasmids from staphylococci. Formation of a DNA hairpin in the pSK41 oriT and the importance of the associated NES Hairpin Loop 1 and 2 had been suggested previously (7). We demonstrate here that NES Hairpin Loops 1 and 2 are important for proper DNA cleavage and strand transfer (Figure 2C and D) but not for DNA binding (Figure 2A). The large increase in DNA strand transfer causing DNA to be ligated before transfer is complete is likely the biggest contributor to the large reduction of plasmid transfer seen when either Hairpin Loop 1 or Loop 2 are eliminated from the encoded NES enzyme (7). It is also likely that accessory proteins in the pSK41 relaxosome complex with NES through interactions with the NES Hairpin Loop 1 and 2, amplifying the effect of loss of these protein features.

Because relaxases are essential for transfer, share many common features, and are unique to the conjugative plasmid system, they represent a novel therapeutic target for decreasing the spread of antimicrobial resistance to allow current antimicrobial compounds to maintain efficacy. As explored previously, there are two potential sites of disruption common to relaxases: the metal binding site and specific protein-DNA interactions (7, 26). These results validate the NES Hairpin Loop 1 and 2 DNA interactions as a target site for such therapeutics. By disrupting the specific protein-DNA interactions in the NES Hairpin Loops, a molecule such as a sequence-specific polyamide could specifically disrupt cleavage and religation during pSK41 conjugation (7). As there seems to be some sequence conservation at the base of
the DNA hairpin, this inhibitor molecule could target mobilizable plasmids in addition to the conjugative plasmid. Interestingly, there is a biological example of relaxase interference from *Staphylococcus epidermidis* strains carrying a CRISPR spacer that matches the *nes* gene of pSK41 and limits conjugative transfer (27). Targeted disruption of conjugation after initiation of the process and formation of the mating pore could cause cell death specifically in conjugative plasmid containing bacteria. This targeted approach to bactericidal compounds is desirable as we learn more about the importance of the human skin microbiome (28).

Despite the importance of the protein-DNA interactions at the DNA hairpin, we show that there is some flexibility in the DNA hairpin sequence allowing sequences from pSK156 and pCA347 to be processed by NES. The origin-of-transfer-mimic sequences of pSK156 and pCA347 maintain all but one or two protein-DNA contacts, respectively, and are able to be bound, cleaved, and ligated by NES, although with altered efficiency. We were therefore somewhat surprised to find that plasmid constructs containing pCA347 or pSK156 ori*T* mimics could not be mobilized from cells harboring pSK41 co-resident, in contrast to a pSK41 *ori*T construct. However, the analogous relaxase-*in trans* mobilization phenomena recently described for the distinct pWBG749-like conjugative plasmids provide a precedent that likely explains this apparent paradox. Namely, pWBG749 *ori*T-like sequences exist as sub-types differentiated by sequence divergence in an inverted repeat (IR2) located adjacent to the *nic* site-containing core sequence (18). This results in specificity between various mobilizable plasmids and particular pWBG749-like conjugative plasmids. Thus, pWBG749 can mobilize plasmids with a pWBG749-like *ori*T of sub-type OT49 but not those carrying an OT45 sub-type, which instead...
can be mobilized by pWBG749-like conjugative plasmids that possess a cognate OT45 sub-type oriT (18). Despite this, pWBG749 was able to stimulate recombination between OT49- and OT45-type oriT sequences carried on the same mobilizable plasmid, indicating that the pWBG749 relaxosome could recognize the OT45-type oriT even though it can’t mediate transfer of that sub-type (18). By analogy, it would seem plausible that the pSK156 and pCA347 oriT-mimics examined here represent sub-types of pSK41-like oriTs that can be recognized by NES but cannot be mobilized by the pSK41 relaxosome. In the case of the pWBG749 system, it has been shown that specificity for IR2 sub-types is dictated by a small putative DNA-binding accessory protein, SmpO, encoded adjacent to the oriT on pWBG749, rather than the relaxase SmpP (18). The involvement of accessory proteins in the pSK41 relaxosome is yet to be established.

Importantly, the scenario proposed above implies the existence of pSK41-like conjugative plasmids with variant oriT sequences that would be capable of mobilizing plasmids such as pSK156, pCA347 and other plasmids listed in Supplemental Dataset S2. Although no such variant pSK41-like plasmids have been detected to date, the presence of variant pSK41-like oriT mimic sequences on one fifth of all sequenced staphylococcal plasmids (not including pSK41-like plasmids themselves) makes the whereabouts of such a reservoir an important question, since it is clearly influencing the evolution of plasmids in clinical staphylococci.

Interestingly, the origin-of-transfer-mimic sequence found in pCA347 is identical to a sequence found in pWBG757, a plasmid that could not be mobilized with pWB749 in the studies by O’Brien and colleagues (17). Comparative data such as this may allow
us to classify mobilizable plasmids into families related to relaxase(s) used for trans-
mobilization. The origin-of-transfer-mimic sequence of pSK156 is also found in
plasmid pWB747, which could be mobilized by pWBG749, suggesting pWBG747
harbors two distinct origin-of-transfer sequences to maximize its ability to be
transferred (17, 18).

We searched the sequenced Staphylococcal plasmids for other pSK41 oriT-like
sequences and found 85 sequences from 83 different plasmids, including 14 pSK41-
family conjugative plasmids (Supplemental Dataset S1). This represents 23.6%
Staphylococcus plasmids which is significantly lower than the 53% of plasmids
O’Brien et al. identified as harboring the pWBG749 oriT-like sequence (18).
However, the set identified here includes 26 plasmids that do not have a pWBG749
oriT sequence, suggesting NES is an important actor in the relaxase-in trans
conjugation mechanism.

Analysis of all 85 sequences shows that oriT sequences identical to that of pSK41
are only evident on plasmids which encode their own NES protein. On other
plasmids, the pCA347 and pSK156 hairpin sequences with their one-nucleotide
difference are by far the most common oriT mimic, representing 73% of the
sequences (Supplemental Dataset S2). The other two major pSK41 oriT mimic types
are significantly different in sequence but are still predicted to form a DNA hairpin
which will allow for most of the NES protein-DNA interactions seen with the pSK41
oriT to be maintained, again suggesting that these specific protein-DNA interactions
may be a potential therapeutic target. It is likely that that NES proteins are capable of
acting on a wide range of non-conjugative staphylococcal plasmids that contain an
oriT mimic sequence, ranging from the oldest known multidrug resistance plasmid pSK156 to prevalent contemporary plasmids such as pMW2 and pUSA300HOU MR. The results described here imply that the recently described relaxase in-trans mechanism of mobilization extends beyond pWBG749-like conjugative plasmids to the clinically more prevalent pSK41-like plasmids, thereby further increasing the proportion of staphylococcal plasmids that are potentially mobilizable. These observations lend further weight to the recent proposal that relaxase-in trans mobilization represents a significant driver of horizontal transfer in staphylococci (18).

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23. The PyMOL Molecular Graphics System, Version 1.5.0.5, Schrödinger, LLC.


**FIGURE LEGENDS**

**Figure 1. Structure of NES Relaxase Domain and pSK41 oriT.**

A. Structure of the NES relaxase domain in complex with DNA from the pSK41 origin of transfer (4HT4, 13). The NES Hairpin Loop 1 is shown in yellow and NES Hairpin Loop 2 in green.

B. Hairpin Loop 1 (yellow) of NES binds in the minor groove of the DNA hairpin formed by the pSK41 origin of transfer while Hairpin Loop 2 (green) binds to the major groove.

C. Schematic of the pSK41 oriT and the oligonucleotides used in these studies. When only black portion is used, the oligo is referred to as OriTHP30. When the sequence is extended to include the orange portion, it is referred to as OriTHP35, the teal as OriTHP40, and the purple as OriTHP45.

**Figure 2. Functional Analysis of NES Loop Deletion Mutants.**

A. $K_D \pm$ standard deviation of DNA binding measured by fluorescence anisotropy for the indicated pSK41 oligonucleotides and NES mutants.

B. Schematic of the DNA cleavage and strand transfer assays. DNA cleavage assays involve only the red DNA substrate labeled with a 5' 6-FAM. DNA strand transfer assays involved both the red and black DNA substrate with the red substrate being unlabeled and the black substrate being 5' 6-FAM labeled.

C. Functional analysis of cleavage activity of NES mutants on varying pSK41 oligonucleotides.

D. Functional analysis of strand transfer activity of NES mutants on varying pSK41 oligonucleotides to mimic religation during conjugative plasmid transfer.
Figure 3. Modeled Structure of the pSK41, pSK156, and pCA347 oriTs.

A. Schematic of the pSK41, pSK156, and pCA347 oligonucleotides used in these studies. Colored nucleotides indicate a difference in sequences from the pSK41 oriT.

B. The relaxase domain of NES in complex with the pSK41 oriT. The box shows the region focused on for Figure 3C, D and E.

C. Contacts between the NES relaxase domain Hairpin Loop 1 and 2 amino acids and the pSK41 oriT nucleotide.

D. Contacts between the NES relaxase domain Hairpin Loop 1 and 2 amino acids and the modeled pSK156 oriT nucleotide. Green nucleotides differ from the pSK41 oriT.

E. Contacts between the NES relaxase domain Hairpin Loop 1 and 2 amino acids and the modeled pCA347 oriT nucleotide. Gold nucleotides differ from the pSK41 oriT.

Figure 4. NES Processing of pSK41, pSK156, and pCA347 oriT Oligonucleotides.

A. $K_D \pm$ standard deviation of DNA binding measured by fluorescence anisotropy for the pSK41, pSK156, and pCA347 oriTs. The pSK41 data is the WT NES data as presented in Figure 2A.

B. Cleavage activity of the pSK41-encoded NES protein on the pSK41, pSK156, and pCA347 oriTs.

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Table 1. Relaxase-*in trans* mobilization of plasmids containing * oriT* sites

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>oriT site</th>
<th>Transfer frequency*</th>
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<tbody>
<tr>
<td>pSK5632</td>
<td>none</td>
<td>9.9 x 10⁻⁵</td>
</tr>
<tr>
<td>pSK6877</td>
<td>pSK41</td>
<td>1.4 x 10⁻⁴</td>
</tr>
<tr>
<td>pSK6879</td>
<td>pCA347</td>
<td>6.6 x 10⁻⁵</td>
</tr>
<tr>
<td>pSK6881</td>
<td>pSK156</td>
<td>8.7 x 10⁻⁵</td>
</tr>
</tbody>
</table>

* Transfer frequencies are presented as per-donor frequencies and are the average of three experiments.
* p < 0.005
** p = 0.0002
relative to WT
**% Cleavage Product Formation**

- **pSK41**
- **pSK156**
- **pCA347**

*** p < 0.0001
Relative to pSK41