Structural and Functional Dissection of the Heterocyclic Peptide Cytotoxin Streptolysin S*

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The human pathogen Streptococcus pyogenes secretes a highly cytolytic toxin known as streptolysin S (SLS). SLS is a key virulence determinant and responsible for the β-hemolytic phenotype of these bacteria. Despite over a century of research, the chemical structure of SLS remains unknown. Recent experiments have revealed that SLS is generated from an inactive precursor peptide that undergoes extensive post-translational modification to an active form. In this work, we address outstanding questions regarding the SLS biosynthetic process, elucidating the features of substrate recognition and sites of post-translational modification to the SLS precursor peptide. Further, we exploit these findings to guide the design of artificial cytolytic toxins that are recognized by the SLS biosynthetic enzymes and others that are intrinsically cytolytic. This new structural information has ramifications for future antimicrobial therapies.

Streptolysin S (SLS) is secreted by the human pathogen Streptococcus pyogenes, the causative agent of diseases ranging from pharyngitis to necrotizing fasciitis (1). SLS is a potent cytolytin that is ribosomally synthesized, extensively post-translationally modified, and exported to exert its effects on the target cell (2, 3). The expression of SLS promotes virulence in animal models of invasive infection and accounts for the hallmark zone of β-hemolysis surrounding colonies of these bacteria grown on blood agar (2, 4). An intriguing feature of SLS is its nonimmunogenic nature (5). This characteristic is likely due to its small size and its capacity to lyse cells involved in both innate and adaptive immunity (6, 7). The β-hemolytic phenotype of S. pyogenes has been studied since the early 1900s, but the molecular structure of SLS has remained elusive (8). In the last decade, transposon mutagenesis studies identified the gene encoding the SLS toxin precursor (sagA, for SLS-associated gene) and eight additional genes in an operon required for toxin maturation and export (9). Targeted mutagenesis of the sag operon yields nonhemolytic S. pyogenes mutants with markedly diminished virulence in mice (2). More recently, it was demonstrated that the protein products of sagA–D are sufficient for the in vitro reconstitution of cytolytic activity (3). The first gene product, SagA, serves as a structural template that after a series of tailoring reactions matures into the active SLS metabolite (see Fig. 1A). A trimercer complex of SagBCD catalyzes these tailoring reactions, which results in the conversion of cysteine, serine, and threonine residues to thiazole, oxazole, and methyloxazole heterocycles, respectively (3).

A DNA gyrase inhibitor, microcin B17, is produced by an orthologous biosynthetic cluster (mcb) found in a subset of Escherichia coli strains (10–12). Microcin B17 contains four thiazole and four oxazoline heterocycles, which are indispensable for biological activity. By analogy to microcin B17 and the lactobiotics, the heterocycles of SLS are formed on the C terminus of SagA, whereas the N terminus serves as a leader peptide (13–15). The installation of thiazole and (methyl)-oxazoline heterocycles restricts backbone conformational flexibility and provides microcin B17 and SLS with rigidified structures. The SLS heterocycles are formed via two distinct steps; SagC, a cyclodehydratase, generates thiazoline and (methyl)-oxazoline heterocycles, whereas SagB, a dehydrogenase, removes two electrons to afford the aromatic thiazole and (methyl)-oxazole (3, 16, 17). SagD is proposed to play a role in trimer formation and regulation (see Fig. 1A). The final genes in the genetic cluster encode a predicted leader peptidase/immunity protein (Sage), a membrane-associated protein of unknown function (SagF), and three ABC transporters (SagGHI).

It is now appreciated that many other prokaryotes harbor similar genetic clusters for the synthesis of thiazole and (methyl)-oxazoline heterocycles (3, 18, 19). Additional important mammalian pathogens such as Listeria monocytogenes, Staphylococcus aureus, and Clostridium botulinum, contain sag-like gene clusters that produce SLS-like cytolsins. These toxins are expected to promote pathogen survival and host cell injury during infection, but this has only been conclusively shown for S. pyogenes and L. monocytogenes (2, 18). Like E. coli, many other prokaryotes harbor a sag-like genetic cluster but are not known to produce cytolsins. Some examples are the gadosporin-pro-
ducing organism, Streptomyces sp. TP-A0584 and cyanobactin producers such as Prochloron didemni (20–22). The molecular targets of these secondary metabolites remain to be elucidated, but it is known that goadsporin exhibits antibiotic activity, and the cyanobactin, patellamide D, reverses multiple drug resistance in a human leukemia cell line (23). Because genetic loci containing sagBCD-like genes have been widely disseminated in prokaryotes (3), nature appears to have found a preferred route to synthesizing such secondary metabolites.

In this work, we build upon our initial report on the in vitro reconstitution of SLS biosynthesis to uncover the requisite features of substrate selectivity and cytolytic activity. The impetus for defining substrate tolerance arose from earlier results showing that SagBCD accepts alternate substrates in vitro (3), as evidenced by two key experiments. First, SagBCD converted a noncognate substrate, CloSA (C. botulinum), into a cytolytic entity. Second, mass spectrometry revealed heterocycle formation on the McbA (E. coli) peptide after SagBCD treatment (3). Here, we dissect the N-terminal leader peptide and C-terminal protoxin of SagA to define the residues necessary for conversion into SLS.

**EXPERIMENTAL PROCEDURES**

**In Vitro Synthetase Reactions**—Protein preparation and synthetase reactions employing maltose-binding protein-tagged substrate and SagBCD were performed as described earlier (3). Membrane damage was quantified by the erythrocyte lysis assay. In every case, omission of the substrate or the SagBCD synthetase resulted in no detectable hemolytic activity (data not shown).

**Cytolytic Activity Assay**—In the in vitro and genetic reconstitution assays described below were performed at least three times for each substrate tested. Because of lot-to-lot variation in commercial blood sources and prep-to-prep variation in the specific activity of the synthetase complex, we have elected to report activity semi-quantitatively. In vitro hemolytic activity equal to wild type SagA treated with SagBCD is reported as three plus signs (+++). Cytolytic activity that is ~30–70% of wild type SagA is given as (++). Detectable activity that is less than 30% of SagA is thus a single plus sign (+), and nondetectable activity is a single minus sign (−). The cytolytic activity of mutant substrates tested via genetic reconstitution was scored in an analogous manner. All of the assays were internally normalized, and the base line was adjusted using two positive controls (Triton X-100 and wild type SagA treated with SagBCD) and two negative controls (substrate and SagBCD alone).

**Transformation and Verification of S. pyogenes M1 sagA Mutants**—The sagA allelic exchange mutant of S. pyogenes M1 was made electrocompetent using a previously published glycine/sucrose method (2). Maxiprepped pDCerm constructs (3 and 12 µg) were incubated with electrocompetent S. pyogenes M1 ΔsagA and electroporated using an Eppendorf 2510 electroporator set to 1.5 kV. These cells (50 and 150 µl) were then plated on Todd-Hewitt agar plates supplemented with 2 µg/ml erythromycin. Typically, 5–15 colonies would appear ~40 h post-transformation. The insert size was evaluated by screening transformants by colony PCR. Clones harboring an appropriately sized insert were initially screened for cytolytic activity by streaking bacteria on blood agar plates (Hardy Diagnostics).

All of the clones were verified to be S. pyogenes (Group A Streptococcus) by using the BBL Streptocard Enzyme Latex Test (BD Diagnostics).

**Cytolytic Assay of Genetically Reconstituted Mutants**—Because of the possibility that nonphysiological concentrations could lead to artifactual activity in vitro, the cytolytic activity of the peptide substrates were also tested using genetic reconstitution. This method requires that endogenous SagBCD accept the substrate. The method described below does not involve lysing the bacteria. Therefore, mutant substrates must also be proteolytically processed and accepted by the SagGHI export apparatus (ABC transporters). Because of toxicity in E. coli and transformation difficulties, intrinsically lytic SagA mutants were not tested by genetic reconstitution. Extracts containing bovine serum albumin-stabilized SLS were prepared in the following manner. Overnight cultures (10 ml) of S. pyogenes M1 ΔsagA containing pDCerm-sagA plasmids were grown to A400 ~0.6 in Todd Hewitt broth containing 2 µg/ml erythromycin. The cultures were treated with bovine serum albumin (10 mg/ml) for 1 h at 37 °C and then centrifuged (6,000 × g, 10 min) before passing the supernatant through a 0.2-µm acrodisc syringe filter (Pall Corporation). These samples were centrifuged again (6,000 × g, 10 min), and the supernatants were assayed for hemolytic activity by addition to defibrinated sheep blood (in V-bottom microtitre plates at 1:25 and 1:50 dilutions). The blood was treated for 2–4 h before assessing hemolytic activity as previously reported (3).

**Assessment of SLS Mutants in a Marine Skin Infection Model**—The experiments were performed using models reported previously (4, 9, 24). S. pyogenes M1 ΔsagA complemented with sagA-WT, sagA-S39A, and sagA-7C/S were grown to log phase, harvested by centrifugation, washed, resuspended in phosphate-buffered saline, and mixed 1:1 with Cytodex beads (1 mg/ml; Sigma). An inoculum of (1 × 107 colony-forming units in 100 µl) of S. pyogenes ΔsagA complemented with either sagA-S39A or the sagA-7C/S mutant was then injected subcutaneously into the right flank of 4-week-old male hairless crl: SKH1(hrhr)Br mice (n = 8/group). S. pyogenes ΔsagA complemented with sagA-wt was injected into the left flank of the same animal for identical comparison. The animals were monitored daily for development of necrotic ulcers. At 4 days post-infection, all of the animals were sacrificed. Biopsies were performed on injection sites for histopathologic assessment (hematoxylin/eosin staining) after measuring the size of tissue ulcers.

**Generation of [35S]Met-SagBCD**—Radioactive synthetase was prepared using maxiprepared pET28-maltose-binding protein-SagBCD and in vitro transcription/translation under T7 promoter control. Rabbit reticulocyte extract (Promega) gave superior yield and purity to wheat germ and S30 extracts. A typical 50-µl reaction was set up as follows: 1–3 µg of plasmid DNA was added to a master mix containing 2 µl of transcription/translation buffer, 25 µl of rabbit reticulocyte extract, 1 µl of RNAsin, 1 µl of minus Met amino acid mix, 1 µl of T7 RNA polymerase, 10 µCi of [35S]Met (PerkinElmer Life Sciences), and DNase/RNase-free water. A small aliquot (0.5 µl) of the radiolabeled product was separated by SDS-PAGE, dried, and...
visualized by autoradiography using a Kodak BioMax low energy isotope intensifying screen (20 h, -80 °C).

Peptide Array—Immobilized peptides were synthesized on cellulose membranes using a MultiPep Autospot synthesis robot following the manufacturer’s instructions (Intavis AG). Irradiation with 254 nm (UV) light gave an indication of the relative amount of peptide per spot. [35S]Met-SagB, -C, and -D were allowed to bind to the array for 15 h at 4 °C in Tris-buffered saline/Tween 20 (0.1% v/v) supplemented with 2.5% non-fat milk and 2.5% bovine serum albumin (both w/w). The array was then extensively washed with Tris-buffered saline/Tween (4 × 10 min, 23 °C) before exposing film with a Kodak BioMax low energy isotope intensifying screen (20 h, -80 °C). Under these conditions, SagB and SagD did not bind tightly to the array, as indicated by a weak radioactive signal, even after longer exposure times (4 days). Using SagBCD together led to a substantial amount of “radioactive precipitation” on areas of the cellulose membrane that did not contain peptide. Therefore, later experiments were carried out using SagC alone.

RESULTS

The SagA Leader Peptide Provides Substrate Recognition—The precursor peptide from C. botulinum, CloA, shares significant amino acid sequence similarity with SagA (62%) and was converted to a cytolsin by recombinant SagBCD (3). The microcin B17 precursor, McbA, is only moderately similar (32%) but was also accepted as a substrate by SagBCD. Therefore, we hypothesized that SagBCD would accept numerous noncognate substrates (Fig. 1B). To demonstrate that the permissive behavior of SagBCD on CloA was not limited to in vitro biochemical studies with purified proteins, genetic complementation studies were performed using a sagA deletion mutant produced in an M1 serotype strain of S. pyogenes (ΔsagA). By transforming this strain with a plasmid encoding the desired peptide substrate, the in vivo selectivity of endogenous SagBCD was probed. β-Hemolysis on blood agar was restored when either the wild type sagA or cloA gene was provided to the S. pyogenes ΔsagA mutant bacteria. To increase sensitivity, we adopted a liquid phase hemolysis assay. Here, the SLS is extracted from the S. pyogenes lipoteichoic acid layer using bovine serum albumin as a carrier/stabilizer. This sample is mixed with sheep blood, and the amount of hemoglobin released from lysed erythrocytes is quantified by absorbance. Using this assay, we confirmed that CloA was converted to a cytolsin by endogenous SagBCD but calculated the level of activity to be reduced by ∼0.5 relative to that of SagA (Fig. 1B).

Interestingly, the related peptide substrates from S. aureus (StaphA) and L. monocytogenes (ListA) did not exhibit detectable hemolytic activity with purified recombinant proteins or in genetically complemented S. pyogenes ΔsagA (Fig. 1B). Cotter et al. (18) have recently shown that the sag-like genetic cluster from L. monocytogenes synthesizes an SLS-like cytolytic factor. Therefore, the lack of activity in our assays must originate at the level of substrate recognition or enzymatic tolerance. Previous work from the Walsh laboratory has shown that the N-terminal leader peptide of McbA is required for substrate recognition (14). The most important residues implicated in this process are McbA-F8 and -L12, which are proposed to lie on the same face of an α-helix (15). Alignment with other substrates shows that a similar motif (FXXXB, where X is any amino acid and B is a branched chain amino acid) is found in the leader peptides of SagA and CloA but is lacking in StaphA and ListA (Fig. 1B). This finding prompted the construction of a double alanine mutant of the FXXXB motif (SagA-FIA) and chimeric substrates comprised of the SagA leader peptide fused to the StaphA and ListA C terminus (Fig. 1C). If the SagA leader peptide contains adequate substrate recognition information, then activity should be restored upon treating the chimera with purified SagBCD. Furthermore, if the FXXXB motif is required for substrate recognition, then cytolytic activity should be reduced for similarly treated SagA-FIA. A full restoration of in vitro cytolytic activity was observed for both the SagA-ListA and SagA-StaphA chimera. In contrast, genetic complementation revealed that only SagA-StaphA retained activity, indicating that either the endogenous rules of cytolytic conversion are more restrictive or the SagA-ListA substrate is not efficiently exported. Enzymatic promiscuity could theoretically be amplified in vitro by the presence of large amounts of purified SagBCD. This is supported by the observation that SagA-FIA has detectable activity using purified enzymes but not under genetic reconstitution conditions (Fig. 1C).

Unnatural SagA analogs were prepared to shed light on the positional requirements of heterocycle formation. We first designed an artificial substrate, SagX, in an attempt to assess the minimum features for the formation of a cytolsin. Four criteria were used to design this potential substrate: (i) the SagA leader peptide to provide recognition, (ii) a stretch of contiguous heterocyclizable residues adjacent to the leader peptide cleavage site, (iii) 30% glycine evenly distributed through the C-terminal half, and (iv) a serine flanked by two glycine residues. Akin to SagA-ListA, this artificial substrate gave activity equal to SagA in the in vitro assay but no activity upon complementation of the ΔsagA mutant of S. pyogenes. The region of SagA between the cysteine-rich region and the last heterocyclizable residue (33FSIA...GSYT50) was further probed by designing two additional unnatural substrates. SagA inversely inverted residues 33–50 of SagA, whereas in SagA scramble this region was reordered in a randomized fashion. Despite having the highest protxin similarity to wild type SagA, the SagA inverse substrate did not yield lytic activity in either assay. SagA scramble was active in vitro but did not complement hemolytic activity in S. pyogenes ΔsagA. These data show that the leader peptide, especially the FXXXB motif, is important for substrate acceptance and that heterocyclizable positions must be available at precise positions to allow creation of an SLS-like cytolytic factor. Additionally, these data indicate that even though SagBCD accepts artificial substrates, there is a limit to the enzymatic promiscuity. These limits are more pronounced during genetic complementation experiments most likely because substrate recognition and enzymatic tolerance are bypassed to an extent when using purified SagBCD. An alternative explanation is that the unnatural substrates are secreted from S. pyogenes with differing efficiencies.

Analysis of SagA Leader Peptide Binding Requirements—Because the above activity assays measure both substrate binding and heterocycle formation in a simultaneous and indirect fash-
ion, peptide arrays were synthesized to directly evaluate binding. Based on a perceived importance of the FXXXB motif in SagBCD substrate acceptance, the first array consisted of a panel of leader peptides that contain the FXXXB motif. Next to each wild type (WT) sequence, the FXXXB double alanine mutation was synthesized for SagA, ClosA, McbA, and a substrate from Pyrococcus furiosus, designated PagA (Fig. 2A).

Equal peptide loading was confirmed by irradiating the array with UV (254 nm) light. To assess binding, [35S]Met-labeled SagBCD was prepared by in vitro transcription/translation (supplemental Fig. S1) and allowed to interact with the peptide array as described under “Experimental Procedures.” Initially, the SagBCD complex was tested for binding in approximately a 1:1:1 ratio, as judged by the number of methionines and the intensity of exposure. Unfortunately, this led to a substantial amount of precipitation that could not be removed from the cellulose membrane and rendered the binding information unreliable. Therefore, SagB, SagC, and SagD were tested individually for binding. SagB and SagD did not bind tightly to the leader peptide array and were washed off before exposing the film (data not shown). SagC, however, bound with high affinity to the array. For the WT sequences, SagC binding was highest to SagA, followed by ClosA and PagA (Fig. 2A). Binding to McbA was the weakest, as measured by autoradiographic intensity. Importantly, binding to each FXXXB mutant was reduced in comparison with the corresponding WT leader peptide.
The FXXXB Leader Motif Is Necessary but Not Sufficient for SagC Recognition—Although the FXXXB motif is an important determinant for directing SagC substrate binding, it does not contribute all of the interaction energy. This is illustrated by the observation that SagC bound to the SagA-WT leader peptide more efficiently than the other WT peptides (Fig. 2A). Furthermore, simple incorporation of FXXXB into the StapA and ListA leader peptide did not provide sufficient affinity to detect SagC binding by this method (data not shown). These observations led to the hypothesis that additional residues of SagA contribute to SagC binding.

To elucidate these binding determinants, a second array was synthesized that consisted of an alanine scan of the SagA leader peptide (Fig. 2B). Each residue of SagA that is not naturally found as alanine was individually mutated to alanine, and tested for SagC binding as above. As expected, mutation to alanine did not disrupt SagC affinity at every location. However, the binding levels were significantly reduced when residues comprising the FXXXB motif were mutated (Phe4 and Ile8). It was also found that residues adjacent to the FXXXB sequence (Leu2, Lys3, and Leu9) and another binding site (Thr17, Gln18, and Val19) were also critical for SagC binding (Fig. 2B).

An additional array was used to ascertain the contribution of each interaction site found in the leader peptide and to also assess whether the C-terminal region of SagA plays a role in binding. A panel of fourteen 10-mer peptides were included in the array that scan the full-length of SagA, except for the oxidatively prone, and synthetically challenging, cysteine-rich region (Fig. 2C). After binding SagC to this array, it was found that only the first peptide was capable of providing enough binding information to retain SagC through the washing steps. Peptide 1 comprises residues 1–10 of SagA and not only contains the FXXXB motif but also the important adjacent residues (Leu2, Lys3, Phe4, Ile8, and Leu9). Peptide 5 contains Ile8 and Leu9 but only the first residue of the TQV site (The17). This peptide is insufficient at retaining SagC. Taken together, these findings demonstrate that separate sites within the N-terminal leader peptide synergize to provide SagC with a high affinity binding site.

Binding parameters for the SagA-WT, SagA-FIA (FXXXB double alanine mutant), and SagA-TQV (triple alanine mutant) leader peptides with SagC were then measured by surface plasmon resonance to quantify the contribution of each binding motif. Using a C-terminal hexa-histidine tagged version of SagA-WT, -FIA, and -TQV, the rates of association (kₐ) and dissociation (kₐ⁻) were first measured with SagC (Table 1). A typical sensorgram and curve fitting analysis are provided in supplemental Fig. S2. As expected from the peptide array data, the effects of FXXXB and TQV mutation were substantial. Compared with SagA-WT, SagC bound to the SagA-FIA peptide ~16-fold less tightly. The reduction in affinity was accounted for by an increased kₐ (kₐ was unchanged). SagC affinity for SagA-TQV was also reduced (~24-fold). The major factor in the SagA-TQV mutant reduction in affinity is an increased kₐ⁻, however, there is also a slight
decrease in $k_a$ (<2-fold) upon comparison with SagA-WT (Table 1).

We next determined whether the rules of substrate recognition applied to other cyclodehydratases. ClosC and McbB, from *C. botulinum* and *E. coli*, respectively, were tested for SagA binding. Relative to SagC, binding to the SagA-WT leader peptide was reduced with both ClosC and McbB (~12- and ~130-fold, respectively; Table 1). This trend correlates with the degree of amino acid similarity to SagC (59% for ClosC; 28% for McbB). Mutation of the SagA FXXXB and TQV motifs led to additional losses of affinity in the same manner as for SagC binding (*i.e.* both the FXXXB and TQV mutants effect $k_a$; the $k_a$ is only slightly affected by the mutation of TQV). This suggests that although each cyclodehydratase is likely fine-tuned for a single endogenous substrate, the substrate binding mechanism is conserved across species.

**Mutational Analysis of Heterocyclizable Residues of SagA—** Numerous attempts at identifying sites of heterocycle formation on SagA by mass spectrometry have failed. As a result, a site-directed mutagenesis approach was initiated to determine functionally important residues of SagA with the assumption that the lack of cytolysis by particular SagA point mutants is suggestive of heterocycle locations. To begin this study, every heterocyclizable residue (cysteines, serines, and threonines) located on the protoxin half of SagA (residues 24–53) was individually mutated. Cysteines and serines were mutated to alanines, whereas threonines were mutated to valines, a closer structural mimic. The lytic activity of these proteins was assessed *in vitro* after treatment with purified SagBCD and through genetic complementation of *S. pyogenes* BCD and through genetic complementation of *S. pyogenes* ΔsagA. Each mutation was classified as having no effect (mutant SLS was fully active), a contributing effect (reduced activity), or a critical effect (inactive). Using these two independent assays, key functional residues of SLS were identified. It is important to note that our method of testing the cytolytic activity of SagA point mutants through genetic complementation measures activity and secretion in a simultaneous fashion.

Compiled results are overlaid on the amino acid sequence of SagA (Fig. 3A) and are also provided in table form (Fig. 3B). This

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**TABLE 1**

**Contribution of SagA Interaction Sites to Cyclodehydratase Binding Kinetics**

Surface plasmon resonance was performed using leader peptides of SagA with a C-terminal His$_6$ tag as the immobilized ligand. The sequences of the peptides are as follows: SagA-WT, MKKTSNLATSLAVETQTVAPGGHHHHHH; SagA-FIA, MKKTSNLATSLAVETQTVAPGGHHHHHH; SagA-TQV, MKKTSNLATSLAVETQTVAPGGHHHHHH; SagA-McbB, AAAA. Contributions of the mean. The error is reported as standard deviation of the mean. $k_a$ is given as an assessment of curve fitting accuracy.

| Ligand | Analyte | $k_a$ ($10^6$) | $k_d$ ($10^6$) | $K_d$ | $\chi^2$ |
|--------|---------|----------------|----------------|-------|----------|
| SagA-WT | SagC    | 3.7 ± 0.4      | 0.25 ± 0.03    | 6.7 ± 0.8 | 0.04     |
| SagA-FIA | SagC   | 3.7 ± 0.3      | 3.5 ± 0.3      | 95 ± 7.0 | 0.04     |
| SagA-TQV | SagC   | 3.1 ± 0.3      | 3.4 ± 0.8      | 160 ± 30 | 0.03     |
| SagA-WT | ClosC  | 3.8 ± 0.3      | 3.1 ± 0.7      | 82 ± 14  | 0.10     |
| SagA-FIA | ClosC | 3.3 ± 0.5      | 7.2 ± 1.0      | 220 ± 30 | 0.07     |
| SagA-TQV | ClosC | 2.5 ± 0.3      | 9.6 ± 0.9      | 380 ± 40 | 0.03     |
| SagA-WT | McbB   | 2.5 ± 0.3      | 22.2 ± 4.0     | 380 ± 140 | 0.58 |
| SagA-FIA | McbB | 2.3 ± 0.2      | 27 ± 1.0       | 1200 ± 100 | 0.25 |
| SagA-TQV | McbB | 2.0 ± 0.3      | 41 ± 5.2       | 2100 ± 310 | 0.04 |

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**FIGURE 3. Identification of SagA residues necessary for cytolytic activity.** A, compilation of the cytolytic data for SagA point mutations after reaction with SagBCD. Residues that are C-terminal to the predicted leader peptide cleavage site (vertical line of SagA 24–30) are shown. Positions that contribute to cytolysis activity are shown in light blue. The positions critical for cytolysis are dark blue and underlined. B, the activity of each SagA mutant was tested by the erythrocyte lysis assay *in vitro* using purified proteins and *in vivo* by genetic complementation. Activity scoring is as in Fig. 1B. Multiple sites were mutated. 2C/S, 23/S1/C32/S; 3C/S, 26/S/C27/S/C28/S; 7C/S, all seven cysteines mutated to serine; 4T/V, all four threonines mutated to valine. C, the intrinsic activity of SagA substituted with proline was tested using purified protein. No SagBCD was used in this assay. Toxicity (tox) was observed for some SagA proline mutants, which prevented recombinant expression. A synthetic peptide was prepared to test proline substitution at each serine found to be important for SagA activity.
analysis revealed that not all sites of potential heterocycle formation were important for activity, but most mutations had a measurable impact on function. Two mutations, C32A and S39A, completely abolished the cytolytic activity of SLS in both assays and are thus highly likely sites of thiazole/oxazole formation. Moreover, it was found that serine and cysteine could substitute for one another at these two critical sites (C32S and S39C) with a small reduction of in vitro activity. However, serine cannot be used to replace multiple adjacent cysteines as indicated by the lack of activity in C31S/C32S (2C/S), C26S/C27S/C28S (3C/S), and a total serine (7C/S) mutant (Fig. 3B). Similarly, valine cannot completely replace threonine (4T/V).

Overall, the activities measured by in vitro reconstitution and genetic complementation agreed. The one case were the assays did not concur was with the C24A mutation. We observed substantial lytic activity in vitro but no activity in genetically complemented bacteria (Fig. 3B). This residue is predicted to flank the leader peptide cleavage site. We have previously shown that leader peptide removal is not required for in vitro activity (3). Thus, a reduction of activity in genetic complementation experiments may result from reduced leader peptide processing and/or secretion. These results confirm that many residues within SagA are important for activity and predict that SLS is highly post-translationally modified.

Proline Can Substitute for Thiazoles and Oxazoles in the SagA Propeptide—We rationalized that because thiazoles and oxazoles are five-membered, nitrogen-containing heterocycles that restrict peptide backbone conformation, proline may be capable of serving as a structural mimic. Therefore, the SagA-C32P and -S39P mutants were prepared and treated with purified SagBCD. These yielded substantial in vitro lytic activity (Fig. 3B) and encouraged the construction of SagA mutants that were cytolytic in the absence of SagBCD. Thus, proline was systematically incorporated into select heterocyclizable sites of SagA that were previously determined to be important for activity (positions 32, 34, 39, 46, 48). This proline-substituted SagA panel was then tested for lytic activity in the absence of SagBCD (Fig. 3C). Wild type SagA and two single substituted proline mutations (C32P and S39P) did not exhibit intrinsic lytic activity. In contrast, two double proline substituted mutants showed dose-dependent activity without SagBCD treatment (C32P/S39P and S34P/S39P) (Fig. 3C and supplemental Fig. S3). A triple mutant, C32P/S34P/S39P, predicted to further enhance lytic activity, killed E. coli upon induction of protein expression and yielded no protein. Likewise, additional permutations revealed that if either Cys32 or Ser34 are substituted in combination with Ser39, Ser46, and Ser48, E. coli toxicity was encountered. To support the claim that toxicity arose because of SLS-like activity, a synthetic peptide comprised of the C-terminal 21 residues of SagA was synthesized with prolines at positions 34, 39, 46, and 48. This peptide, although devoid of cysteine, was intrinsically lytic in a dose-dependent manner (Fig. 3C and supplemental Fig. S3). The observation that select proline mutations endow SagA with intrinsic lytic activity bolsters the conclusion that the above sites are heterocyclized in SLS.
strate tolerance of SagBCD was initially assessed using substrates from other members of the firmicutes phylum that were suspected of producing SLS-like cytolsins. To begin, we chose the substrate from *C. botulinum*, which harbors the most highly related *sag*-like genetic cluster (*cloSA-I*) known outside of the *Streptococcus* genus. SagBCD accepted CloSA as a noncognate substrate in *vitro* and in genetically complemented *S. pyogenes* ΔsagA bacteria, but the activity was reduced relative to SagA (Fig. 1A). This could stem from a number of possibilities. The most likely explanation, in light of our binding data, is that SagBCD process CloSA more slowly because of a lowered substrate affinity. *In vitro*, CloSA treated with purified SagBCD resulted in activity equal to SagA. However, under the conditions employed, nonphysiological concentrations of synthetase and substrate could easily result in an elevated level of substrate permisivity. Other explanations for reduced CloSA activity upon genetic reconstitution could be that on a molar basis SLS is more potent or that the leader peptidase and/or export machinery process the toxin more slowly. If the latter were true, toxicity would be an expected outcome, but analysis of growth curves indicated that this is not the case (data not shown).

Because CloSA and McbA were both known *in vitro* substrates of the SagBCD synthetase (3), we were initially surprised when SagBCD rejected potential substrates more similar to SagA than McbA (i.e. StaphA and ListA; Fig. 1B). However, chimeric substrates consisting of the SagA leader peptide coupled with the StaphA or ListA pro toxin were fully active *in vitro* after treatment with recombinant SagBCD. This indicated that the SagA leader peptide conferred substrate recognition to SagBCD. Later experiments showed that a partially conserved motif in the SagA leader peptide, FxxxB, played a major role in efficient cytolytic conversion (Fig. 1C) and SagC affinity (Fig. 2 and Table 1). Additionally, the SagA leader peptide can be fused to properly designed artificial pro toxins to yield cytolsins after treatment with SagBCD.

Peptide array binding experiments uncovered that in addition to the FXXXB motif and some adjacent residues, SagC also recognizes another site (TQV) on the SagA leader peptide (Fig. 2). Given that the *Kd* was measured to be in the low nanomolar range, multiple binding sites on SagA would be presumed necessary to drive such an avid interaction (Table 1). Mutation of the SagA-TQV site led primarily to an increased *k*d with a modest decrease of *k*s relative to SagA-WT (Table 1). This is in contrast to the SagA-FIA mutant, which only led to an increased *k*d. These binding trends also held for the SagA binding interaction with the cyclodehydratases from a closely related organism (*C. botulinum*, CloSA) and a distinctly related organism (*E. coli*, McbB; Table 1). We hypothesize this relationship will be true of all *sag*-like clusters that harbor substrates with FXXXB motifs in the leader peptide.

Elucidation of the SagC substrate recognition requirements was undertaken to judge why certain substrates are accepted by the SagBCD synthetase and why others are not. Earlier studies have demonstrated that the SLS precursor from a fish pathogen, *Streptococcus iniae*, was able to complement the hemolytic activity of *S. pyogenes* ΔsagA (25, 26). Upon comparison with SagA from *S. pyogenes*, the *S. iniae* leader peptide has a single mutation in the leader sequence (K3Q). Thus, it should not be surprising that SagA from *S. iniae* was an efficient substrate of *S. pyogenes* SagBCD. We conclude that ClosA was accepted by recombinant and endogenous SagBCD because of a complete FXXXB site (LKF and VL) and a partial TQV site (Val19). Previous results has also shown that SagBCD accepts McbA as a substrate (3). This is supported by the present work demonstrating that SagC directly binds to the McbA leader peptide (Fig. 2A). Relative to SagA, the SagC binding to McbA was greatly reduced, but the data provide a satisfactory explanation; the leader peptide of McbA contains partial SagC-binding sites. The FXXXB sequence is present, but the important surrounding residues are nonconservatively mutated. Also, the Glu of the TQV site is present (Fig. 2A).

The identification of the TQV site helps explain why simple inclusion of FXXXB into the StaphA and ListA leader peptides did not result in high affinity SagC binding. Residues adjacent to the FXXXB motif (LKF and IL) also contribute to this interaction. Of a total 23 residues in the predicted SagA leader peptide, eight are directly involved in driving substrate affinity. This implies that upon SagC binding SagA, nearly the entire length of the leader peptide will be associated, possibly buried, in a SagC-binding site. Undoubtedly, the extended recognition capability of SagC acts to prevent the synthetase complex from processing unwanted peptides/proteins. Perhaps this affinity is also a protective measure employed by *S. pyogenes*. Our work has demonstrated that pro-SLS (Fig. 1A) is highly active, and it is possible that a tightly bound synthetase complex will cage the activity until the metabolite is ready for export.

SLS has historically been recalcitrant to isolation and characterization. However, the early work of Bernheimer (27, 28), Bernheimer and Schwartz (29), and Koyama (30–32) shed important light on the nature and action of SLS. Using gel filtration analysis on partially purified SLS, Bernheimer (33) predicted that the molecular weight of the polypeptide would be 2.8 kDa. This is quite remarkable, given that a modern calculation of 2.7 kDa is based on modifications to SagA, a peptide discovered over 30 years later. Our results suggest that in the mature structure of SLS, oxazoles will be formed from SagA residues Ser39, Ser36, Ser48, and Ser49 (Fig. 3B). The work of Kolter and co-workers (10–12) on microcin B17 provides a literature precedent for heterocycle formation when the preceding residue is glycine (Ser39, Ser36, and Ser49) (34, 35). Presumably, the increased flexibility in the peptide backbone at these locations facilitates the orbital alignment required for cyclodehydration. Oxazole formation at the abovementioned sites is further supported by the creation of proline-substituted SagA mutants that exhibit intrinsic cytolytic activity (Fig. 3C).

Our mutagenesis data confirm a previous study identifying SagA Cys24 and Cys27 as residues important for the activity of SLS on blood agar plates (2). Despite this, the fate of the cysteine-rich region (Cys24–Cys27) remains speculative. Given the observation that C32S and C32P were cytolytic, we expect Cys32 to be converted to a thiazole. However, serine cannot simultaneously substitute for multiple adjacent cysteines. This implies that there is either a polythiazole-specific electronic contribution to cytolsis or that another type of modification is present in the mature structure of SLS. Our current data cannot distinguish between these two possibilities, which will require
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extensive methodology improvements. We hypothesize that it is unlikely free thiols, disulfides, or lanthionines will be present in the final SLS structure. This is because SLS is known to be oxygen-stable (36, 37), and cysteine was not detected in an amino acid analysis study (30). Moreover, the sagA-I cluster, which is sufficient to confer SLS production to a heterologous bacterial species (4), does not harbor the appropriate dehydratases and cyclases to form lanthionine linkages (4, 38, 39).

The murine infection study supports our previous data that SLS heterocycle formation is required for the virulence of *S. pyogenes*. Furthermore, we show that a single amino acid change, SagA-S39A, renders this pathogen avirulent in a skin infection model (Fig. 4). Without active SLS, the bacteria change, SagA-S39A, renders this pathogen avirulent in a skin infection model (Fig. 4). Without active SLS, the bacteria exhibit undetectable pathogenicity and are efficiently cleared by the murine immune system. It is conceivable that in such a setting, antibodies could be raised against inactive SLS mutants. Whether this represents a viable strategy for vaccine development remains to be seen, but with the tactical role SLS-like toxins play in pathogenesis, further study is warranted.

This study has elucidated the requisite features of SagBCD substrate recognition and SagA residues of functional importance. We conclude that although the C terminus of SagA undergoes enzymatic conversion, only the N-terminal leader peptide substantially contributes to substrate recognition. Conceivably, these principles could be used to guide the design of other artificial toxins for antibiotic or anticancer applications. Additionally, our study has yielded a wealth of structure/activity data on a conserved family of bacterial virulence factors and lays the foundation for the development of a structure-based vaccine.

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