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An *E. coli*-based bioengineering strategy to study Streptolysin S biosynthesis

Andrew L. Markley¹, Emily R. Jensen², and Shaun W. Lee^{2,†}

¹Department of Chemistry and Biochemistry, University of California at San Diego, La Jolla, CA, 92093

²Department of Biological Sciences, Eck Institute for Global Health, University of Notre Dame, Notre Dame, IN, 46556

Abstract

Group A *Streptococcus pyogenes* (GAS) is a leading human pathogen that produces a powerful cytolytic bacteriocin known as Streptolysin S (SLS). We have developed a bioengineering strategy to successfully reconstitute SLS activity using heterologous expression in lab strains of *E. coli*. Our *E. coli*-based heterologous expression system will allow more detailed studies into the biosynthesis of other bacteriocin compounds, and the production of these natural products in much greater yield.

Keywords

Bacteriocin; Bioengineering; Streptolysin S; Natural Products

Group A *Streptococcus pyogenes* (GAS) is a leading human pathogen that can progress to dramatic invasive syndromes such as necrotizing fasciitis and toxic shock syndrome[1]. A hallmark of GAS infection is a clear zone of hemolysis surrounding colonies grown on blood agar media. The ability of GAS to lyse blood cells is due to a powerful cytolysin known as Streptolysin S (SLS)[2; 3]. SLS production by GAS results in infections with invasive and severe outcomes[4]. Furthermore, recent studies have shown that Streptolysin S can inhibit neutrophil recruitment and impair immunity[5; 6]. SLS has also been implicated in facilitating transepithelial spread of GAS[7]. These findings demonstrate that SLS is likely to contribute to the pathogenesis of GAS infections by multiple strategies, making it a critical virulence factor for study. Genetic analyses have shown that SLS biosynthesis is mediated by a nine-gene cluster (*streptolysin S-associated gene, sag*) in the genome of GAS[8].

Although attempts to identify the structure of the natural toxin Streptolysin S have not been successful, we have previously demonstrated the first *in vitro* reconstitution of the activity of streptolysin S, the substance responsible for the characteristic pattern of hemolysis produced by GAS[9]. Using the purified forms of the precursor peptide and modifying complex from *S. pyogenes*, we have demonstrated that SLS is a small, ribosomally produced bacteriocin-like toxin that undergoes heterocyclic addition at specific residues to confer hemolytic

[†]To whom correspondence should be addressed. lee.310@nd.edu.

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activity. Our *in vitro* studies revealed that three candidate enzymes, SagBCD, are necessary and sufficient to convert the SLS precursor Sag A into a hemolytic molecule. The biosynthetic gene cluster that is responsible for the synthesis of SLS is highly conserved and widespread, and its presence spans multiple phyla, including Cyanobacteria and Euryarchaeota[9]. Although SLS is a potent toxin and an important human virulence factor, other related heterocyclic peptide toxins such as Microcin B17 have unique bacterial targets[10; 11]. Thus, many of these undiscovered heterocyclic compounds, classified as bacteriocins, are likely to be important and powerful antibiotics that bacteria use to establish and maintain their environmental niche. It is likely that the discovery of similar peptidic antibiotics will rapidly expand as more genomes are sequenced. Importantly, these new peptide antibiotics are produced ribosomally, and thus are amenable to genetic engineering strategies. Finally, many gene clusters resembling Microcin B17 and SLS are present in microorganisms that are difficult to isolate, maintain, and study[12; 13]. Therefore, heterologous approaches using model organisms such as *E. coli* would offer a marked advantage in studying the biosynthetic process behind many of these novel bacteriocin-like compounds.

Gene complementation studies using *Streptococcus pyogenes* have demonstrated that insertional inactivation of each of the nine genes located in the Sag gene cluster can be complemented *in trans* using gene complementation methods[14]. However, previous data have shown that not all of the complemented genes can restore wild type levels of toxin activity using this approach. These limitations therefore make it difficult to utilize such approaches to perform detailed studies, such as assessing the role of particular domains in overall function of the SLS synthetases. Heterologous expression of various components of the SLS biosynthetic gene cluster have been attempted. Nizet et al. cloned the entire nine-gene region of the Sag gene cluster and introduced a plasmid containing this region into the non-hemolytic *Lactococcus lactis*[8]. Using the pSagLocus plasmid, Nizet demonstrated that the nine-gene *sag* cluster was sufficient to confer a significant amount of hemolytic activity when introduced heterologously into *Lactococcus lactis*. However, a major limitation of this method is the inability to control the expression of the individual genes that are introduced into the non-native host. Indeed, introduction of the pSagLocus plasmid into the Gram-negative host *E. coli* did not result in hemolytic activity as reported by the same authors. This is likely due in part to the incompatibility of promoters between the Gram-positive Group A *Streptococcus* and the Gram-negative *E. coli*.

To overcome the existing limitations with heterologous expression of SLS and other bacteriocins from divergent microorganisms, we describe herein an *E. coli*-based multi-gene expression system for the biosynthesis of the SLS toxin. This system allows for all of the genes necessary in the natural product biosynthesis pathway to be individually added as independent open reading frames into the expression host plasmid. All of the genes necessary for the production of the natural product can be added or removed by means of simple cloning procedures into robust *E. coli* expression sites. Our expression system also allows mutagenesis studies to be rapidly performed by PCR-based methods, because the genes are located on easily isolated plasmids. Finally, our heterologous expression system can be utilized to rapidly insert similar genes from other similarly produced bacteriocins, such as those from the family of thiazole-oxazole modified microcins (TOMMs)[15]. Importantly, our *E. coli* based heterologous expression method will be especially applicable for investigating the biosynthesis of ribosomally derived compounds from microorganisms for which genomes have been sequenced, but for organisms that have been difficult to culture in sufficient yields for study.

We developed a multi-plasmid based expression strategy as a means to successfully reconstitute SLS activity by heterologous expression in lab strains of *E. coli* using a

pETDUET based protein expression system. This is a pET-derived *E. coli* expression system that contains two unique cloning sites for the simultaneous expression of two genes off of a single plasmid. The pETDUET expression system can be extended such that up to four plasmids can be introduced into a single bacterium, each with an independent origin of replication and two cloning sites. In this manner, up to eight proteins can be expressed simultaneously under inducible control. This system has been used previously in other systems to characterize protein complexes in eukaryotic systems[16; 17].

The overall organization of the plasmids used for heterologous reconstitution of SLS in *E. coli* is depicted in Figure 1. Our previous *in vitro* reconstitution studies demonstrated that the precursor protein SagA, and the synthetase complex SagBCD, are necessary and sufficient to convert the SagA precursor into an active hemolysin. Thus we reasoned that cloning of these four genes into pETDUET vectors would drive sufficient expression of the minimal products necessary to produce a functional SLS-like toxin in *E. coli*. These plasmids, designated pETDUET-1-SagBC and pACYCDuet-1-SagDA were transformed into *E. coli* expression strain BL21-DE3 (Invitrogen). As a safety measure, antibiotic selection pressure ensures that SLS production by *E. coli* is reversed when antibiotics are not present in growth media.

To test the ability of pETDUET-SLS *E. coli* to produce a hemolytic molecule, we induced *E. coli* containing SagABCD using isopropyl- β -D-thiogalactopyranoside (IPTG); BSA was added as a stable carrier protein for active SLS. Supernatants were processed for SLS activity measurement by a microtiter hemolysis assay. Figure 2A demonstrates levels of hemolytic activity present after protein induction and SLS production by *E. coli*. SLS activity from *E. coli* supernatants was also strictly concentration dependent, as characteristic of an active hemolysin. Taken together, these findings represent the first heterologous expression of SLS activity using *E. coli*-based bioengineering methods.

One substantial advantage of our bioengineering strategy is the ability to rapidly screen enzyme mutants for activity in a high-throughput fashion. We performed mutagenesis studies to examine the importance of putative conserved residues present in the amino acid sequence of the synthetase enzyme SagC. Based on sequence homology of SagC in *Streptococcus pyogenes* to SagC-like genes in the closely related pathogens *Streptococcus iniae*, *Staphylococcus aureus*, and *Clostridium botulinum*, we probed the functional importance of several candidate residues using our pETDUET-SLS system. Quikchange PCR mutagenesis was used to generate point mutants in SagC (Invitrogen). Figure 2B shows a partial alignment of SagC amino acid sequences from *Streptococcus pyogenes*, *Streptococcus iniae*, *Staphylococcus aureus*, and *Clostridium botulinum*, and depicts the conserved residues that were probed for function. Hemolytic assays performed on supernatants of pETDUET-SLS SagC mutants demonstrated that mutation of many of these conserved residues greatly lowered SLS-toxin production (Figure 2C). Importantly, these initial findings demonstrate that our pETDUET-SLS system can be used to quickly probe structure/function relationships that can be investigated further by biochemical approaches.

Summary

We have reported here the first successful multi-plasmid system for the expression of the SLS biosynthetic gene cluster in *E. coli*. This method holds promise for investigating the biosynthesis of ribosomally-derived compounds from microorganisms that are difficult to culture in sufficient yields for natural product extraction and identification. This system will allow researchers to probe a vast chemical space in a high-throughput manner by producing libraries of enzyme combinations and unnatural precursor peptides. We are currently using

this system to explore the potential of the pETDUET *E. coli* system to produce a library of novel heterocyclic bacteriocins to screen for medically relevant, active compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- [1]. Carapetis JR, Steer AC, Mulholland EK, Weber M. The global burden of group A streptococcal diseases. *Lancet Infect. Dis.* 2005; 5:685–694. [PubMed: 16253886]
- [2]. Nizet V. Streptococcal beta-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol.* 2002; 10:575–580. [PubMed: 12564994]
- [3]. Bernheimer AW. Physical behavior of streptolysin S. *J. Bacteriol.* 1967; 93:2024–2025. [PubMed: 6025314]
- [4]. Humar D, Datta V, Bast DJ, Beall B, De Azavedo JC, Nizet V. Streptolysin S and necrotising infections produced by group G streptococcus. *Lancet.* 2002; 359:124–129. [PubMed: 11809255]
- [5]. Lin A, Loughman JA, Zinselmeyer BH, Miller MJ, Caparon MG. Streptolysin S inhibits neutrophil recruitment during the early stages of *Streptococcus pyogenes* infection. *Infect. Immun.* 2009; 77:5190–5201. [PubMed: 19687200]
- [6]. Miyoshi-Akiyama T, Takamatsu D, Koyanagi M, Zhao J, Imanishi K, Uchiyama T. Cytocidal effect of *Streptococcus pyogenes* on mouse neutrophils in vivo and the critical role of streptolysin S. *J. Infect. Dis.* 2005; 192:107–116. [PubMed: 15942900]
- [7]. Sumitomo T, Nakata M, Higashino M, Jin Y, Terao Y, Fujinaga Y, Kawabata S. Streptolysin S contributes to group A streptococcal translocation across an epithelial barrier. *J. Biol. Chem.* 2011; 286:2750–2761. [PubMed: 21084306]
- [8]. Nizet V, Beall B, Bast DJ, Datta V, Kilburn L, Low DE, De Azavedo JC. Genetic locus for streptolysin S production by group A streptococcus. *Infect. Immun.* 2000; 68:4245–4254. [PubMed: 10858242]
- [9]. Lee SW, Mitchell DA, Markley AL, Hensler ME, Gonzalez D, Wohlrab A, Dorrestein PC, Nizet V, Dixon JE. Discovery of a widely distributed toxin biosynthetic gene cluster. *Proc. Natl. Acad. Sci. U S A.* 2008; 105:5879–5884. [PubMed: 18375757]
- [10]. Heddle JG, Blance SJ, Zamble DB, Hollfelder F, Miller DA, Wentzell LM, Walsh CT, Maxwell A. The antibiotic microcin B17 is a DNA gyrase poison: characterisation of the mode of inhibition. *J. Mol. Biol.* 2001; 307:1223–1234. [PubMed: 11292337]
- [11]. Yorgey P, Lee J, Kordel J, Vivas E, Warner P, Jebaratnam D, Kolter R. Posttranslational modifications in microcin B17 define an additional class of DNA gyrase inhibitor. *Proc. Natl. Acad. Sci. U S A.* 1994; 91:4519–4523. [PubMed: 8183941]
- [12]. Schmidt EW, Nelson JT, Rasko DA, Sudek S, Eisen JA, Haygood MG, Ravel J. Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinium patella*. *Proc. Natl. Acad. Sci. U S A.* 2005; 102:7315–7320. [PubMed: 15883371]
- [13]. Sudek S, Haygood MG, Youssef DT, Schmidt EW. Structure of trichamide, a cyclic peptide from the bloom-forming cyanobacterium *Trichodesmium erythraeum*, predicted from the genome sequence. *Appl. Environ. Microbiol.* 2006; 72:4382–4387. [PubMed: 16751554]
- [14]. Datta V, Myskowski SM, Kwinn LA, Chiem DN, Varki N, Kansal RG, Kotb M, Nizet V. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Mol. Microbiol.* 2005; 56:681–695. [PubMed: 15819624]

- [15]. Melby JO, Nard NJ, Mitchell DA. Thiazole/oxazole-modified microcins: complex natural products from ribosomal templates. *Curr. Opin. Chem. Biol.* 2011
- [16]. Small E, Eggler A, Mesecar AD. Development of an efficient *E. coli* expression and purification system for a catalytically active, human Cullin3-RINGBox1 protein complex and elucidation of its quaternary structure with Keap1. *Biochem. Biophys. Res. Commun.* 2010; 400:471–475. [PubMed: 20735986]
- [17]. Tolia NH, Joshua-Tor L. Strategies for protein coexpression in *Escherichia coli*. *Nat. Methods.* 2006; 3:55–64. [PubMed: 16369554]

Figure 1.

Overview of the SLS expression cluster and expression strategy. **A.** Architecture of the Group A *Streptococcus* SLS expression operon. *sagA* encodes the substrate peptide SLS precursor, *sagB*, *sagC*, and *sagD* encode the heterocyclic modification enzymes for SLS maturation. **B.** Flowchart depicting cloning strategy for pETDUET-SLS plasmid construction and gene expression. Overall organization of the plasmids used for heterologous reconstitution of SLS in *E. coli* is indicated. The genes were inserted into the pETDUET expression plasmid sequentially either by subcloning (*SagA* and *SagB*) or PCR amplification (*SagC* and *SagD*).