Published in final edited form as:

Nat Rev Microbiol. ; 9(9): 670–681. doi:10.1038/nrmicro2624.

Streptolysin S-like virulence factors: the continuing saga

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Abstract

Streptolysin S (SLS) is a potent cytolytic toxin and virulence factor produced by nearly all *Streptococcus pyogenes* strains. Despite a 100-year history of research on this toxin, it has only recently been established that SLS represents the archetypal example of an extended family of post-translationally modified virulence factors also produced by some other streptococci and Gram-positive pathogens, such as *Listeria monocytogenes* and *Clostridium botulinum*. In this Review we describe the identification, genetics, biochemistry and various functions of SLS. We also discuss the shared features of the virulence-associated SLS-like peptides, as well as their place within the rapidly expanding family of thiazole/oxazole-modified microcins (TOMMs).

Introduction

Humans are the natural host and sole reservoir of group A *Streptococcus pyogenes* (GAS) (for classification, see ¹⁻²). The organism can survive and replicate in a variety of body locations including the skin, throat and blood ³, and is a common cause of illness, especially in children. The more typical infections include self-limiting skin disorders such as impetigo and respiratory tract infections such as pharyngitis. In rare cases, complications can lead to destructive soft tissue infections including necrotizing fasciitis and the multisystem disorder of streptococcal toxic shock syndrome ³. In recent decades, a dramatic increase in severe invasive GAS infections has been documented worldwide ⁴⁻⁵. These infections carry a significant risk of mortality ⁶⁻⁷ with an estimated 500,000 deaths worldwide, most of which are attributed to invasive infection or acute rheumatic fever and subsequent rheumatic heart disease ⁸⁻⁹.

Although the ability of certain strains of streptococci to haemolyse red blood cells (beta-haemolysis) ¹⁰⁻¹² was first observed as early as 1895 ¹³⁻¹⁴, it was not until 1938 that Streptolysin S (SLS) was identified as being one of two distinct toxins responsible for the ability of GAS to lyse mammalian erythrocytes ¹⁵; the other being the unrelated, large cholesterol-dependent, oxygen-sensitive toxin, Streptolysin O (SLO) ¹⁶⁻¹⁷. Although SLS has been subject to rigorous investigation (Box 1), isolation of the mature SLS toxin and elucidation of its molecular structure has proven elusive ¹⁸.

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SLS is a 2.7 kDa ribosomally synthesised, post-translationally modified peptide that is regarded as the prototypical member of a distinct group of *Streptococcus*-associated toxins whose haemolytic activity is abolished by the addition of trypan blue¹⁹. It is extensively modified, resulting in the formation of distinctive heterocycles, prior to export²⁰⁻²¹. In addition to an unusual structure, SLS is cytolytic only when associated with the bacterial cell surface or in the presence of certain “carrier” molecules²²⁻²⁴. The cytolytic spectrum of the toxin is broad and includes erythrocytes, leukocytes, platelets and sub-cellular organelles²⁵⁻²⁸, but excludes bacteria with intact cell walls²⁹. SLS is not immunogenic in the course of natural infection³⁰, which may reflect both its small size, highly modified nature (which removes proteolytic sites that are critical for antigen digestion/display) and, perhaps most importantly, its potent cytotoxicity against cells involved in both innate and adaptive immunity³¹⁻³². While the exact mechanism of SLS toxicity is not yet fully understood, it has been suggested that its accumulation in cell membranes results in the formation of transmembrane pores and irreversible osmotic lysis³³.

It is becoming apparent that SLS represents the founding member of a class of post-translationally modified virulence peptides, with homologous toxins now identified in other *Streptococcus* species. Furthermore, analysis of genomic data has identified gene clusters that are similar to the SLS-associated cluster in other disease-causing pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus*. In this Review we describe the identification of SLS, the GAS chromosomal locus that encodes SLS and the proteins involved in its production, as well as the initial steps towards characterisation of its structure. We provide a brief overview of the role of SLS in virulence and other putative functions. We discuss the identification of SLS-like loci in non-GAS streptococci and the advances in identification of SLS-like loci in other genera. Finally, we highlight the recent discovery that similar biosynthetic clusters are found in numerous microbial phyla and the subsequent definition of a new family of ribosomally-produced peptides i.e. thiazole/oxazole-modified microcins (TOMMs).

The GAS SLS-associated gene cluster

The chromosomal locus responsible for SLS production was first identified following the characterisation of transposon mutants of GAS that did not produce SLS³⁴⁻³⁵. The transposons disrupted the promoter for a gene, designated *sagA* (SLS-associated gene), that encodes the 53-amino acid SLS precursor, SagA. Subsequent chromosome-walking studies and genome-sequence data resulted in the identification of a contiguous nine-gene locus (*sagA* to *sagI*), which was found to constitute an operon³⁶⁻³⁷ (Figure 1). Further analysis confirmed that the *sag* operon encodes all the accessory proteins required for proper processing and export of SLS³⁶.

Following an *in silico* analysis of the *sag* operon, it became apparent that SLS is related to the bacteriocin family of antimicrobial peptides or, more specifically, to a number of post-translationally modified (Class I) bacteriocins^{36,38}. Class I bacteriocins are encoded by an operon that contains a structural gene for a precursor peptide with an N-terminal “leader” region and C-terminal “core” peptide³⁹. The operon also includes genes encoding the machinery required for post-translational modification of the core peptide, as well as leader cleavage and export of the mature form of the bacteriocin. In addition to their antimicrobial activity, in rare cases modified bacteriocins also exhibit broader haemolytic and cytolytic properties⁴⁰, as is the case with the cytolysis produced by *Enterococcus faecalis*⁴¹.

SagA possesses features reminiscent of bacteriocin precursor peptides, such as a potential Gly-Gly leader cleavage site that would yield a 23 aa leader peptide and a 30 amino acid structural peptide⁴²⁻⁴³. This structural peptide contains an abundance of residues that are

frequently the target of post-translational modification in bacteriocins, including serines, threonines, cysteines and glycines⁴³. Site-directed mutagenesis of conserved residues in SagA supports its designation as a bacteriocin-like toxin²⁰, as does the fact that SagA shares features reminiscent of McbA³⁶, the precursor of the bacteriocin microcin B17 of *Escherichia coli*⁴⁴. Furthermore, in common with many bacteriocin operons, a 'leaky' rho-independent terminator sequence after *sagA* acts as a regulator of transcript abundance³⁶ (Figure 1A).

The *sagA* gene is followed by *sagBCD*, genes that exhibit low sequence identity to *mcbBCD* located in the *E. coli* *mcb* gene cluster²¹ (Figure 1B). McbBCD form an enzyme complex required for the post-translational modification of McbA⁴⁵⁻⁴⁶. The predicted protein product of *sagB* is a 36 kDa species that shares some similarity with the McbC dehydrogenase (22% identical), SagC (40.3 kDa) is 13% identical to the McbB cyclodehydratase and SagD is a 51.6 kDa "docking" protein (18% identical to McbD)^{21,36}. Due to these similarities, it was hypothesised that SagBCD might function in a similar manner to the McbBCD synthetase complex, which converts four serines and four cysteines within McbA into oxazole and thiazole heterocycles, respectively⁴⁷. These modifications are essential for the activity of mature microcin B17⁴⁸. It was subsequently established that SagBCD is also capable of catalysing heterocycle formation when recombinant SagBCD successfully substituted McbBCD to process McbA *in vitro*²¹.

Further studies with SagBCD established that the complex is indeed responsible for the conversion of SagA into SLS²¹. Separate sites within the N-terminal leader peptide provide SagC with a high affinity substrate binding site, leading to efficient modification of the structural peptide by the SagBCD complex⁴⁹. The modifications involve the conversion, via two distinct steps, of cysteine, serine, and threonine residues to thiazole, oxazole, and methyloxazole heterocycles, respectively (Figure 2)²¹. SagC removes water from the peptide backbone to produce thiazoline and (methyl)-oxazoline rings, followed by a dehydrogenation reaction catalysed by SagB that formally removes hydrogen to generate aromatic thiazole and (methyl)-oxazole heterocycles²¹. SagD is proposed to have a role in SagBCD complex formation and the regulation of enzymatic activity²¹. The incorporation of these heterocycles into the unmodified precursor peptide restricts backbone conformational flexibility and provides the mature SLS with a more rigid structure. Rigidification is essential for bioactivity, as unstructured peptides must pay too large an entropic penalty to bind efficiently to molecular targets⁵⁰.

Recent research has yielded increased information regarding the structure-activity relationships of SLS, but the precise chemical structure of mature SLS has yet to be fully elucidated. Site-directed mutagenesis approaches with alanine (to reduce backbone rigidity) and/or proline (which retains rigidity) suggest that oxazoles are formed at residues Ser34, Ser39, Ser46, and Ser48, that a thiazole is formed at Cys32⁴⁹, and that Cys24 and Cys27 are also important residues with respect to haemolytic activity^{20,49}. As the incorporation of thiazoles or (methyl)-oxazoles results in a net loss of 20 Da in peptide mass, LC-MS/MS with *in vitro* modified SLS has been employed to provide evidence of heterocycle formation by SagBCD and to confirm the incorporation of oxazole moieties at Ser46 and Ser48⁵¹.

The role of the remaining Sag proteins is less clear. SagF (26.2 kDa) is predicted to be membrane-associated^{21,52} and SagE (25.4 kDa) is expected to be a membrane-spanning peptidase²¹. Although it is reasonable to suggest that SagE might be the enzyme responsible for leader cleavage, it has also been noted that intact *sagE* is required for viability and that the corresponding protein shares weak homology with a candidate bacteriocin immunity (self-protection) protein, PlnP, of *Lactobacillus plantarum*²⁰. SagG (41.7 kDa), SagH (42.2 kDa), and SagI (41.7 kDa) are thought to be membrane proteins that form an (ABC)-type

transporter²⁰⁻²¹ similar to those frequently involved in bacteriocin export^{36,53}, with SagG displaying the signature ATP-binding pocket motifs²⁰.

SLS-like loci in other streptococci

The production of SLS-like cytolysins is not an exclusively GAS-associated trait (Table 1). Such activity is also associated with invasive human isolates of the beta-haemolytic group C and G streptococci, which belong to the streptococcal species *S. dysgalactiae* subsp. *equisimilis*. Homologues are also present in the animal pathogens *Streptococcus iniae* and *Streptococcus equi*.

Group C *Streptococcus* (GCS) is a relatively common cause of acute pharyngitis among young adults⁵⁴⁻⁵⁵. The normally commensal group G *Streptococcus* (GGS) can also cause acute pharyngitis⁵⁶ and has been associated with necrotising soft tissue infections in patients with underlying medical conditions⁵⁷. Both GCS and GGS display a hallmark beta-haemolytic phenotype on blood agar⁵⁸, which has been linked to the action of SLS-like cytolysins⁵⁷. The GCS and GGS *sagA* genes are identical to each other and the corresponding peptides share 89% identity with the GAS SagA⁵⁷. Other conserved features include the *sag* operon promoter, a *rho*-independent attenuator located downstream of *sagA*, as well as eight genes resembling the remaining SLS associated genes⁵⁷. Targeted mutagenesis of the GGS *sagA* gene has been found to abolish beta-haemolytic activity, a phenotype that is partially restored upon transformation of the mutant with the GAS homologue⁵⁷.

S. iniae is a beta-haemolytic pathogen of commercial fish species that can also be a rare human pathogen⁵⁹⁻⁶¹. Characterisation of the genomic region responsible for the haemolytic phenotype of *S. iniae* identified a 9-gene locus that has 73% sequence similarity to that in GAS⁶². Furthermore, heterologous expression of *sagA* from *S. iniae* restored haemolytic activity to GAS Δ *sagA*⁶³. Finally, *S. equi* is the causative agent of “strangles”, a prevalent and highly contagious disease of horses⁶⁴. While an associated *sag* cluster has not yet been identified in this species, the haemolytic activity of *S. equi* has been characterized and found to be caused by a SLS-like toxin⁶⁵.

Functions of SLS

A recent genetic screen of clinical GAS samples has shown that 99% of all isolates are SLS-positive, while the remaining 1% are not predicted to produce SLS and are assumed to be non-haemolytic (Lowry-type) strains⁶⁶. Although these atypical strains have occasionally been associated with human infection⁶⁶⁻⁶⁸, there is little doubt that SLS has an important role in *Streptococcus* pathogenicity. Unsurprisingly, the specific mechanisms by which SLS contributes to virulence have been the subject of much investigation. These proposed mechanisms fall into three categories, namely soft tissue damage, an impact on host phagocytes and a contribution to GAS translocation across the epithelial barrier. SLS also functions as a signalling molecule, and it has been speculated that SLS may contribute to iron acquisition (Figure 3). In the latter case it has been suggested that SLS production could provide a means *via* which GAS could gain access to intracellular iron through the lysis of host red blood cells⁶⁹⁻⁷⁰.

Role of SLS in tissue injury

In vivo studies have demonstrated that SLS is an important virulence factor in skin and soft tissue infection, where it contributes to tissue injury²⁰. The virulence of a SLS-negative GAS mutant is substantially reduced in a murine soft-tissue infection model³⁵. Further studies confirming the importance of SLS with respect to GAS and GGS

pathogenesis^{57,71-73} have revealed that even a single point mutation predicted to interfere with heterocycle formation can render a strain of GAS avirulent in a murine model of skin infection⁴⁹. The direct toxicity of SLS toward cells of the deep soft tissues and feeding vessels, leading to cell death and provoking neutrophil influx, is thought to promote the development of necrotising fasciitis⁵⁷. It has been suggested that other virulence factors, such as SLO²⁰, the anti-phagocytic M surface protein^{20,74-75}, as well as host neutrophil-derived oxidants and proteases^{24,76-78}, interact synergistically with SLS to accelerate necrosis. It has also been established that expression of SLS is required for *S. iniae*-induced local tissue necrosis⁶²⁻⁶³.

Role of SLS in phagocytic clearance

The role of SLS in the resistance of GAS to phagocytic clearance was first uncovered when it was revealed that a $\Delta sagA$ mutant did not survive as well as wild-type GAS in killing assays with human whole blood and purified neutrophils²⁰. It was subsequently revealed that cell-associated SLS actively destroyed neutrophils recruited to the site of infection⁷⁹. The elimination of neutrophils may be a specific virulence mechanism that effectively inactivates the phagocytic cells that are primarily responsible for the ingestion and killing of GAS, thus allowing them to evade the innate immune system⁷⁹. In support of this hypothesis, a paucity or lack of neutrophils is regarded as an unfavourable prognostic sign in patients suffering from necrotizing fasciitis⁸⁰ and in primates who fail to survive GAS-associated necrotizing fasciitis and myositis⁷⁸. Macrophages constitute another crucial component of the host phagocytic defence against GAS infection. It has been established that GAS can kill macrophages, through the activation of an inflammatory programmed cell death pathway mediated by SLS and SLO⁸¹. It is important to note, however, that the relative contribution of SLS to phagocyte resistance is subject to both species and strain variation^{62-63,72-73}, and as such, other factors may also play a prominent role.

GAS is known to produce virulence factors that diminish the host's immune response to infection⁸²⁻⁸⁴. SLS is thought to contribute by affecting the host cell's ability to produce signals that are chemotactic for neutrophils⁸⁵. Using a zebrafish model, it was shown that, in contrast to wild-type GAS, an SLS- mutant was significantly less virulent and was associated with a more robust recruitment of neutrophils⁸⁵.

Finally, little is known about the entry and subsequent multiplication of *S. equi* following the exposure of a susceptible equine host⁸⁶, but data that indicates that virulence is as a result of a potent anti-phagocytic effect and the failure of innate immune defences⁸⁷ suggests that its SLS-like toxin may play an important role.

Role of SLS in GAS translocation

Systemic dissemination of GAS involves bacterial colonisation of the pharynx or damaged skin, followed by penetration of the epithelial barrier. The mechanisms underlying the adherence of GAS to epithelial cells and subsequent internalization have been extensively studied⁸⁸⁻⁹¹. Most internalized GAS are eliminated by intracellular killing. However, it has been speculated that the programmed cell death of epithelial cells induced by massive adherence or internalization of GAS may reduce the barrier function of the epithelium, providing GAS with access to deeper tissues *via* an intracellular route⁹²⁻⁹⁴. GAS tissue invasion *via* a paracellular route has been also been noted⁹⁵ and, significantly, recent research has identified SLS as a critical factor in this process⁹⁶. SLS does this by recruiting the host cysteine protease calpain to the plasma membrane by an as yet uncharacterised mechanism, and then utilises proteolytic activity to degrade intercellular junctions to allow invasion *via* a paracellular route⁹⁶.

SLS as a signalling molecule

Many species of bacteria use a cell density-dependent response system, or quorum sensing, to regulate gene expression⁹⁷. Indeed, several virulence genes in GAS are modulated by quorum sensing⁹⁸⁻⁹⁹. *luxS* is essential for the production of AI-2 of the autoinducer II (AI-2) pathway in a diverse range of bacterial species¹⁰⁰ and, after the identification of a *luxS* homologue in GAS¹⁰¹, a GAS *luxS* mutant was found display a number of altered phenotypes¹⁰². Notably, the SLS activity of the mutant was found to be enhanced as a result of increased *sagA* transcription. As expression of *sagA* is normally induced at high cell density, these results suggested that the AI-2 pathway was not the only element of population-dependent control but rather indicates that *luxS* interacts with a second regulatory pathway to influence its sensitivity¹⁰².

Indeed, since it was first established that SLS expression increases with cell density³⁵, it was suspected that SLS itself might act as a quorum sensing molecule¹⁰³. Several bacteriocins are known to be regulated by quorum sensing³⁸ and in some instances, as is the case for nisin, the structural peptides also function as signalling molecules and induce their own expression upon activation of the density-dependent autoinduction loop¹⁰⁴. Significantly, it has since been established that expression of *sagA* is up-regulated by exposure to SLS¹⁰³. Furthermore, global regulatory functions have been attributed to the *sagA* gene itself, resulting in the designation of *pel*, for ‘pleiotropic effects locus’, to the ORF^{36,105-109}. More specifically, it has been suggested that *sagA* mRNA is involved in the pre- and post-translational control of other virulence factors of the human pathogen, including M proteins, the expression of capsule (SpeB), and streptokinase¹⁰⁸⁻¹¹⁰. In contrast to these results, Datta *et al.* found that elimination of *sagA* does not produce significant pleiotrophic effects²⁰, suggesting that the role of *sagA*/SagA/SLS in the regulation of other virulence factors may differ in a strain variable manner.

SLS-like loci in other bacterial genera

Until recently, post-translationally modified virulence peptides have rarely been reported and SLS-like cytotoxins have been exclusively associated with the genus *Streptococcus*. As noted above, however, many similarities exist between the SLS and the MccB17 biosynthetic operons present in some *E. coli*. This observation prompted a search of public genomic databases in the belief that other prokaryotes might use related machinery to introduce Ser/Thr/Cys-derived heterocycles into a wider variety of ribosomally produced peptides^{21,111}. From this approach, it became evident that a number of Sag-like gene clusters are present in some of the most notorious Gram-positive pathogens, including *L. monocytogenes*, *C. botulinum* and *S. aureus*^{21,111} (Table 1 & Figure 4). In addition, more distantly related clusters were identified in an even more diverse collection of bacteria²¹. This led to the definition of a new class of compounds, characterised by a biosynthetic gene cluster that encodes a small precursor peptide and three adjacent synthetase proteins that serve to introduce thiazole and (methyl)-oxazole heterocycles onto the ribosomally-produced protoxin scaffold. These bioactive natural products and their biosynthetic gene clusters are now referred to as thiazole/oxazole-modified microcins (TOMMs)^{21,50}. Although the biological purposes of the majority of newly identified TOMMs have not been uncovered, it is likely, based on the similarity to MccA and SagA, that some will act as either DNA gyrase inhibitors or membrane-damaging agents¹¹².

Within TOMM-associated gene clusters, the genes encoding the SagB-like dehydrogenase, SagC-like cyclodehydratase, and SagD-like “docking” protein are often found as adjacent open reading frames (ORFs). It is probable that this will aid in the identification of additional orthologous clusters as more genome sequences become available. However, the associated TOMM structural genes are frequently overlooked during annotation because of

their small and hyper-variable nature^{111,113}. Furthermore, although short ORFs encoding proteins of 50–70 residues that are rich in Cys/Ser/Thr are usually found in organisms with *sagBCD*-like genes as adjacent ORFs^{21,111}, TOMM peptides are sometimes situated far from the genes encoding the thiazole/(methyl-)oxazole-forming machinery and in such cases it can be difficult to assign the substrate for a particular TOMM pathway¹¹⁴. The situation is further complicated by the observation that numerous substitutions in the C-terminal core sequence of the propeptide can often be tolerated⁴⁹. This can result in the production of numerous similar peptide products^{50,115–117} by “natural combinatorial biosynthesis”. In addition, new families of more unusual modifying enzymes are often not sufficiently related to characterised relatives to be identified by BLAST searching^{21,118–119}.

Several factors can increase confidence in the designation of an ORF as a TOMM precursor, including sequence similarity to previously identified TOMM precursors, the presence of a suitable leader peptide cleavage motif and a C-terminal core region rich in heterocyclizable residues^{21,49} (Figure 4). The detection of genes encoding enzymes involved in thiazole/oxazole synthesis, dehydroalanine production¹²⁰ and peptide macrocyclization^{113,115,119,121} provides further support when annotating post-translationally modified peptide biosynthetic clusters. The identification of a TOMM cluster is also facilitated by the tendency of the modification enzymes to cluster with other genes associated with the cleavage and export of the final product^{21,36,46}.

It is clear that the extent of structural variation and biological impact of TOMM clusters is gaining greater appreciation. Thus, it is likely that novel bioinformatics-based approaches such as those reported by Haft *et al.*, whereby multiple highly sensitive profile-based search models are used to analyse large numbers of sequenced genomes^{50,114}, will be applied to uncover additional members of this family of modified peptides, as well as helping to identify cognate modification and export genes.

Listeria monocytogenes: Listeriolysin S

L. monocytogenes is an intracellular pathogen that is usually transmitted to humans through contaminated food products¹²². It has a high mortality rate in pregnant women, neonates and individuals with a compromised immune system¹²³. Strains of *L. monocytogenes* are divided into three evolutionary lineages¹²⁴, with lineage I (which consists of strains of serotype 1/2b and the notorious ‘epidemic’ serotype 4b) contributing to the majority of sporadic cases and epidemics associated with this often fatal pathogen^{125–126}.

Since it was first established that Listeriolysin O (LLO)-negative mutants of *L. monocytogenes* did not lyse blood cells, it was believed that this cholesterol-dependent virulence factor was the only cytotoxin produced by *L. monocytogenes*^{127–128}. Recent *in silico* analysis identified a gene cluster in a number of *L. monocytogenes* strains that resembles the *sag* operon and was designated the Listeriolysin S (LLS) gene cluster¹¹¹. The LLS structural gene within this cluster, *llsA*, encodes a peptide consisting of a N-terminal leader region and C-terminal core region with an extreme predominance of cysteine, serine and threonine residues, as well as a putative Ala-Gly leader cleavage motif¹¹¹. As the predicted *llsA* promoter (PllsA) was found to be induced only under oxidative stress conditions, constitutive expression of the operon was used to establish that these genes did indeed encode a SLS-like cytolysin¹¹¹. Like SLS, LLS was found to be active in a cell-associated form but inactive in cell-free situations in the absence of a stabilizer¹¹¹. It is now apparent that previous detection of LLS haemolysis was hindered by the absence of the LLS cluster from the majority of the most frequently used laboratory strains, coupled with the inducible nature of PllsA and the masking effect of LLO activity.

With respect to the rest of the LLS operon, the products of *llsB* (42 % similarity to SagB), *llsY* (37 % similarity to SagC) and *llsD* (46 % similarity to SagD) are predicted to form a synthetase complex necessary for the production of mature, active LLS^{111,129}. LLS is believed to be post-translationally modified in a similar manner to SLS, supported by the fact that a SagA-LlsA chimera (SagA leader sequence fused to LlsA core peptide) is converted into a cytolytic entity by SagBCD *in vitro*⁴⁹. Other proteins encoded by the operon include LlsGH, two components of an ABC transporter (represented by three ORFs, *sagGHI*, in the *sag* operon), and LlsP, which has been annotated as a CaaX protease with 36% similarity to SagE^{111,129-130}. The *llsX* gene is not homologous to any known gene and, as such, is used as a target for a real-time PCR assay designed to identify LLS-positive strains of *L. monocytogenes*¹²⁹. Deletion mutagenesis has established that of the seven genes located downstream of *llsA* in the *lls* operon (i.e., *llsG-llsP*), all except *llsP* are essential for LLS activity^{111,129}.

LLS contributes to pathogenesis as evidenced by significantly reduced levels of a LLS-negative mutant in the livers and spleens of mice following intraperitoneal inoculation¹¹¹. Furthermore, wild-type *L. monocytogenes* survives significantly better than the LLS-negative mutant in purified human polymorphonuclear neutrophils (PMNs). Notably, PMNs are crucial for the resolution of *L. monocytogenes* infections¹³¹. Given the oxidative-stress inducible nature of PllsA and the contribution of LLS to neutrophil endurance, enhancement of the survival of cells still retained in the phagosome upon phago-lysosomal fusion has been proposed as a mechanism by which LLS contributes to virulence¹¹¹. As a consequence of the contribution of the LLS-encoding TOMM cluster to the virulence potential of producer strains, it was named *Listeria* pathogenicity island 3 (LIPI-3) in order to distinguish it from previously identified *Listeria*-associated pathogenicity islands¹³²⁻¹³³. Given that LIPI-3 is consistently absent from all lineage II and III *L. monocytogenes* and that relatively few lineage I outbreak-associated strains lack LIPI-3, it has been postulated that this discovery may well provide the long sought-after explanation for the enhanced virulence of a proportion of lineage I *L. monocytogenes*¹¹¹.

Staphylococcus aureus RF122: Stapholysin S

It is noteworthy that a *sag*-like cluster is also present in the genome of *S. aureus* RF122^{21,111}, a pathogenic strain responsible for bovine mastitis¹³⁴. Of the known *sag*-like clusters, it is most closely related to the *lls* cluster^{21,49,111}. The putative structural gene again encodes a peptide consisting of a proposed N-terminal leader region and C-terminal precursor peptide with an abundance of cysteine, serine and threonine residues, as well as an Ala-Gly suspected leader cleavage site^{21,111}. Thus, it was predicted that mature “Stapholysin S” would act as a cytolyisin²¹. This hypothesis was experimentally validated when a chimeric substrate comprised of the SagA leader peptide fused to the StaphA C-terminus was converted into a cytolytic entity by SagBCD *in vitro*⁴⁹.

Clostridium botulinum/sporogenes: Clostridiolysin S

Using comparative genomic analysis, a nine-gene SLS-type cluster was identified in the genomes of clostridia, including the biological warfare-associated pathogen *C. botulinum* and food pathogen *C. sporogenes*⁵¹. *C. botulinum* is an anaerobic, spore-forming rod that causes the potentially fatal neuro-paralytic diseases of food-borne-, infant-, wound- and inhalation-botulism as well as other invasive infections through the elaboration of potent neurotoxins¹³⁵. *C. botulinum* strains that do not produce botulinum toxin are referred to as *C. sporogenes*¹³⁶.

Before the functionality of the *C. botulinum* SLS-like gene cluster had been confirmed, *in vitro* production of the bioactive natural product *via* SagBCD modification supported its

designation as a TOMM, as did investigations revealing the haemolytic nature of the biotoxin generated²¹. This is not unexpected in light of the organization and amino acid sequence of proteins present in the *C. botulinum* cluster (*closA-I*), which corresponds closely with *sagA-I*. In fact, *C. botulinum* harbours the most highly related *sag*-like genetic cluster known outside of the streptococci⁴⁹. The cytolytic gene product of *closA* has been named Clostridiolysin S, or CLS⁵¹. The functional equivalence of the clostridial SLS-like gene clusters to the SLS biosynthetic pathway was confirmed by the complementation of targeted GAS SLS-operon knockouts with the corresponding *Clostridium* genes⁵¹. Furthermore, the availability of the complete genome sequence of *C. sporogenes*¹³⁷ and the identification of a CLS cluster led to the use of the safer *C. sporogenes* for mutagenesis studies, which established that this gene cluster was indeed responsible for the biogenesis of a haemolytic toxin⁵¹.

A more in-depth investigation of the *clos* genes further highlights the TOMM-associated features. The first gene in the operon, *closA*, encodes the inactive precursor peptide that is post-translationally modified by the synthetase complex, ClosBCD, to form the mature biotoxin⁵¹. ClosG, H, and I are ABC transporters and therefore likely constitute the system required for exporting the mature haemolytic product. ClosE has been annotated as an immunity protein but, like SagE, is similar to the CaaX protease super family¹³⁰ and so might be responsible for leader cleavage from the post-translationally modified propeptide⁵¹. ClosF is a protein of unknown function.

The *in vitro* reconstitution of CLS activity allowed direct confirmation by mass spectroscopy that the non-toxic precursor, ClosA, is post-translationally modified by the synthetase enzymes to contain heterocyclic moieties⁵¹. A heterocycle was identified at position Thr46 within ClosA and the dramatically negative impact of substitution of an alanine at Thr46 on haemolytic activity confirmed the importance of heterocyclic conversion at this site⁵¹. These investigations represent the first step towards the elucidation of the structure of mature CLS.

Other related clusters

The research of Lee *et al.* first highlighted the fact that in addition to the Gram-positive pathogens discussed previously, similar TOMM clusters are widely disseminated in the genomes of a diverse group of microorganisms spanning six phyla²¹. Indeed, even very distantly related microbes, such the thermophilic archaeon *Pyrococcus furiosus*, can contain heterocycle-forming synthetases²¹. In addition to the SLS-like toxins with established or suspected roles in virulence described above, some examples of other TOMM clusters of note are mentioned (Table 1). As a consequence of the recent expansion of the TOMM family, it now encompasses such diverse bacterial products as the microcins¹³⁸, thiazolylpeptides¹³⁹⁻¹⁴⁰ and cyanobactins¹⁴¹. Emerging TOMM subfamilies include *Bacillus*-associated putative thiazole-containing heterocyclic bacteriocins¹¹⁴, as well as a group of nitrile hydratase and NifH-related precursor peptides⁵⁰.

A gene cluster identical in arrangement to that responsible for microcin B17 production has been found in the plant symbiont *Pseudomonas putida* KT2440¹³⁸ and it is speculated that it too functions as an inhibitor of DNA gyrase²¹. Indeed, many other prokaryotes harbour a *sag*-like genetic cluster that is not associated with the production of a cytotoxin, such as the goadsporin-producing microorganism, *Streptomyces* sp. TP-A0584¹⁴². The molecular targets of this secondary metabolite have yet to be elucidated, but it is known that goadsporin promotes secondary metabolism and sporulation in actinomycetes, as well as exhibiting antibiotic activity^{112,120,143}. Other diverse functions encoded by TOMM peptides include the inhibition of ribosomal protein synthesis¹⁴⁴ by the thiazolylpeptides, a

family of more than fifty bactericidal antibiotics¹⁴⁵⁻¹⁴⁶, and reversal of multiple drug resistance in a human leukaemia cell line by the cyanobactin, patellamide D¹⁴⁷⁻¹⁴⁸.

Lastly, the genome of *B. amyloliquefaciens* FZB42, a Gram-positive, plant-growth promoting saprophyte that can suppress the growth of bacterial and fungal plant pathogens¹⁴⁹, was recently shown to contain a novel TOMM cluster²¹. Subsequent investigations have revealed that this unique TOMM, plantazolicin, functions as a narrow-spectrum antibacterial compound¹⁵⁰. It has been suggested that the biological role of this natural product might be to suppress the growth of closely related competitors within the plant rhizosphere¹⁵⁰.

It is apparent that these ribosomally-produced peptide natural products represent a largely hidden arsenal of active small molecules, which, although grouped as a consequence of possessing the same general modifications, can perform a wide variety of functions other than contributing to pathogenicity.

Conclusion

With the ongoing discovery and analysis of biosynthetic genes associated with the production of SLS-like cytolytic peptides, evidence is rapidly mounting that these pathways are both more common and more diverse than previously suspected⁵⁰ and can contribute substantially to the virulence potential of pathogenic bacteria¹⁵¹. Of the myriad of novel putative TOMM-encoding clusters identified, those associated with *L. monocytogenes*, *C. botulinum/sporogenes* and *S. aureus* are particularly noteworthy as a consequence of their similarity to the *sag* cluster and the notoriety of the associated pathogens.

The number and variety of potential producers of SLS-like toxins, and by extension TOMMs, coupled with the fact that secondary metabolites produced by similar biosynthetic clusters have been an abundant source of pharmaceuticals in the past, suggests that further research into this group of peptides will lead to the identification of novel targets for antibiotic and vaccine development. Vaccine or chemotherapeutic strategies designed to neutralize SLS activity could be of benefit as adjuncts to surgical and antibiotic management of human streptococcal infection. The observations that synthetic peptides corresponding to the SLS precursor retain sufficient features of the mature toxin to raise neutralizing antibodies^{33,152}, suggest that subunits or inactivated toxoids of each haemolysin could have significant immunogenicity. Insights gained through the *in vitro* application of the SagBCD biosynthetic machinery and the SagA peptide⁴⁹ could be also used to guide the design of artificial toxins with a view to structure-based vaccines. Furthermore, because targeting virulence factors is becoming an attractive anti-infective strategy, selective inhibition of the family of TOMM synthetase enzymes may be of value.

In summary, the structural diversity and biological impact of both the SLS-like peptide group and the wider TOMM family of natural products is just beginning to be fully appreciated⁵⁰. The proven success of *in silico* analyses suggests that further studies of this type will help to uncover novel TOMM (and other natural product) secondary metabolite biosynthetic systems and will aid in the definition of the evolutionary routes and inter-relationships of the rapidly expanding TOMM ribosomal peptide group. Such studies inform structural investigations into natural products and pave the way for combinatorial biosynthetic studies and rational engineering.

Acknowledgments

Related work in the authors' laboratories are/have been supported by the Irish Government under the National Development Plan and by Science Foundation Ireland (SFI) in the form of the SFI-funded Centre for Science,

Engineering and Technology (SFI-CSET); the Alimentary Pharmabiotic Centre (APC), SFI Principal Investigator funding to PC/CH/RPR and CH/RPR, the Irish Research Council for Science Engineering and Enterprise Ireland. EM has received travel-related funding from the Society for General Microbiology and SFI. The work of DAM is supported by the Department of Chemistry & Institute for Genomic Biology (UIUC).

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Glossary

<i>Streptococcal pharyngitis</i>	Inflammation of the pharynx also known as “strep throat”.
<i>Impetigo</i>	An acute and highly contagious infection of the surface layers of the skin characterised by blisters, pustules and yellowish crusts
<i>Necrotizing fasciitis</i>	A rare but very severe type of soft tissue infection, which can be caused by GAS, that can destroy the muscles, skin, and underlying tissue. It develops when the bacteria enter the body, usually through a minor cut or as a complication of surgery. The mortality rate is high, even with aggressive treatment and powerful antibiotics
<i>Streptococcal toxic shock syndrome</i>	A rare but extremely severe infection that usually presents in people with pre-existing skin infections with GAS and has a very high mortality rate. It is characterised by hypotension and shock. Other symptoms can include kidney impairment, abnormality in blood clotting ability, acute respiratory distress syndrome, rash and local tissue destruction
<i>Beta-haemolysis</i>	Complete red blood cell lysis that appears as yellowing and transparency around and under colonies grown on blood agar media, a phenotype which is routinely used as a diagnostic tool for GAS identification. Beta haemolysis is primarily dependent on SLS, with SLO making a minimal contribution
<i>Streptolysin O</i>	SLO is a thiol-activated, approximately 57 kDa cytolysin produced by group A, C, and G streptococci that is inhibited by small amounts of cholesterol. As a result of its oxygen-labile nature, SLO is most often responsible for haemolysis under the surface of blood agar, while the oxygen-stable SLS results in the zone of clearing surrounding colonies on the surface of blood agar. SLO is antigenic, resulting in anti-SLO antibodies that are useful for documenting recent exposure to GAS

<i>Trypan blue</i>	A defining feature of SLS-like peptides is the fact that they are completely inactivated by trypan blue, a vital stain that is usually used to selectively colour dead tissues, or cells, blue.
<i>Heterocyclic compound</i>	A compound that has atoms of at least two different elements within its ring structure(s). With respect to bioorganic chemistry, heterocycles contain one or more carbon atoms and at least one ring member other than carbon.
<i>SLS “carrier” molecules</i>	The addition of certain high molecular weight “carrier” molecules, such as non-ionic detergents, albumin, α -lipoprotein, lipoteichoic acid and the RNAase-resistant fraction of yeast RNA (RNA core), can stabilize the haemolytic activity from a SLS-producing growing culture or resting cell suspension. SLS is irreversibly inactivated on separation from the carrier or on destruction of the carrier
<i>Chromosome-walking</i>	A technique used for identifying and characterising regions of DNA by the sequential isolation of overlapping sequences of DNA, starting with a known fragment of DNA.
<i>Bacteriocin</i>	A small, ribosomally synthesised, heat-stable peptide produced by one bacterium that is active against other bacteria, either in the same species (narrow spectrum), or across genera (broad spectrum).
<i>Class I bacteriocins</i>	Antimicrobial peptides that undergo extensive post-translational modification to yield their active form. Although this term is most frequently applied to the lantibiotic (i.e. lanthioine-containing) family of bacteriocins, it can be extended to include other post-translationally modified bacteriocins, including microcin B17.
<i>Microcin B17</i>	A ribosomally synthesized antibacterial peptide produced by strains of <i>Escherichia coli</i> that is active, after post-translational modification, against closely related bacterial species. The intracellular target of this natural product is the essential enzyme DNA gyrase.
<i>Lowry-type strains of GAS</i>	In 1971, James and McFarland reported the isolation of a completely non-haemolytic strain of GAS from an outbreak of rheumatic fever at Lowry Air Force Base, Colorado. As a result, these atypical strains are often referred to as “Lowry-type”
<i>Intra- and Paracellular Invasion</i>	Translocation of pathogens across an epithelial barrier can occur via an intracellular route (within the host cells) or via a paracellular route (between the host cells).
<i>Quorum Sensing</i>	A mechanism of communication between bacteria that requires the production and secretion of a signalling molecule, which, when present at or above a critical threshold concentration, induces changes in gene expression in neighbouring cells.
<i>Thiazole/Oxazole-Modified Microcins (TOMMs)</i>	Structurally and functionally diverse family of ribosomally produced peptides with post-translationally installed heterocycles derived from cysteine, serine and threonine residues. These

*Natural
combinatorial
biosynthesis*

modifications rigidify the precursor peptide to endow biological function on the mature natural product

Natural combinatorial biosynthesis is a phenomenon whereby Nature's existing biosynthetic machinery has been repurposed by a particular organism for the production of a library of related compounds. In the case of some TOMM biosynthetic pathways, a single enzyme complex processes numerous substrates that have a common recognition motif, but variable structural C-termini. One striking example is the cyanobactin family of natural products.

Box 1**History of SLS Research**

The SLS-associated beta-haemolytic phenotype of GAS has been subject to rigorous investigation for over a century.

Figure 1. Overview of the production, processing and export of SLS and microcin B17

The C-terminal core peptide of SagA (depicted in red; A), produced by GAS, and McbA (depicted in orange; B), produced by *E. coli*, serve as structural templates that undergo a series of post-translational tailoring reactions by the SagBCD and McbBCD biosynthetic complexes to form biologically active SLS (cytotoxin) and microcin B17 (DNA gyrase inhibitor), respectively. The N-terminal leader (depicted in black) is cleaved from the mature core peptide following modification, resulting in a mature peptide product. Also shown is the 'leaky' rho-independent terminator sequence between *sagA* and *sagB*, and *mcbA* and *mcbB*, that acts as a regulatory mechanism yielding a stoichiometric excess of structural gene transcripts versus a substoichiometric, catalytic amount of transcripts for the modification and transport machinery.

Figure 2. Post-translational modification of SagA via the combined activity of a cyclodehydratase (SagC) and dehydrogenase (SagB) from within a three protein complex (SagBCD)

SLS heterocycles are formed via two distinct steps: SagC, a zinc-tetrathiolate containing cyclodehydratase, removes water from cysteine, serine, and threonine residues in the peptide backbone to generate thiazoline and (methyl)-oxazoline rings. Subsequently, a flavin mononucleotide-dependent dehydrogenation reaction catalysed by SagB removes hydrogen to generate the aromatic thiazole and (methyl)-oxazole heterocycles. An example of each heterocyclizable residue is shown for illustrative purposes, with glycine included as a typical residue that is found N-terminal to cyclized residues to facilitate the orbital alignment required for cyclodehydration^{45,50,153-154}.

Figure 3. Summary of the functions of SLS

The mechanisms *via* which SLS contributes to the virulence of GAS and other functions.

Figure 4. Top Panel: Amino acid sequence of the unmodified precursors of Staphylysin S (StsA), Listeriolysin S (LlsA), Streptolysin S (SagA) and Clostridolysin (BtsA)

The (predicted) leader regions are to the left and terminate in (putative) Gly-Gly/Ala-Gly leader cleavage sites (blue). Residues potentially involved in modification of the precursor peptides are indicated in red (cysteine), orange (serine), green (threonine) and blue (glycine). Based on the sequence of *sagA* and assuming cleavage after the Gly-Gly site, the molecular weight of modified SagA is estimated to be approximately 2.7 kDa. (although this does not preclude the possibility that mature SLS is an assemblage of modified SagA peptides).

Bottom Panel: SLS-like gene clusters in *S. aureus*, *L. monocytogenes*, *S. pyogenes* and *C. botulinum* are depicted. Related genes are indicated by colour. A: structural gene; B: dehydrogenase; C: cyclodehydratase; D: “docking” protein; E/P: CaaX protease; G, H, I: ABC-type transporter components; Z, X, F: proteins of unknown function. In the case of RF122, the genomic map appears to be fragmented, giving multiple ORFs for several biosynthetic proteins.

TOMMs referred to in this review

Table 1

Organism	Disease associated with organism	TOMM	Genes	TOMM function	References
TOMMs in pathogenic bacterial species					
<i>Streptococcus pyogenes</i>	respiratory tract infections, skin diseases, invasive infections	Streptolysin S	<i>sagA-I</i>	haemolytic exotoxin	15,34,36
<i>S. dysgalactiae</i> subsp. <i>equisimilis</i>	pharyngitis, invasive infections	Streptolysin S	<i>sagA-I</i>	haemolytic exotoxin	57
<i>S. iniae</i>	fish pathogen, rare human pathogen causing invasive infection	Streptolysin S	<i>sagA-I</i>	haemolytic exotoxin	62
<i>S. equi</i>	horse pathogen	Streptolysin S	ND	haemolytic exotoxin	65
<i>Listeria monocytogenes</i>	gastroenteritis, septicaemia, meningitis	Listeriolysin S	<i>lisa-X</i>	haemolytic exotoxin	21,111
<i>Clostridium botulinum</i> , (<i>Clostridium sporogenes</i>)	botulism poisoning	Clostridiolysin S	<i>btxA-I</i>	haemolytic exotoxin	21,51,111
<i>Staphylococcus aureus</i> RF122	bovine mastitis	Staphylolysin S	<i>ssaA-Z</i>	haemolytic exotoxin	21,49,111
TOMMs in non-pathogenic bacterial species					
<i>Escherichia coli</i>	NA (gut commensal / pathogen)	Microcin B17	<i>mcbA-G</i>	antibacterial: DNA gyrase inhibitor	44,46-47
<i>Prochloron</i> sp.	NA (photosynthetic endosymbiont of the ascidian <i>Lissoclinum patella</i>)	Patellamide D	ND	reversal of multiple drug resistance in a human leukaemia cell line	147-148
<i>Streptomyces</i> sp. TP-A0584	NA (soil inhabitant)	Goadsporin	<i>godH-I, godR</i>	antibiotic activity, promotion of secondary metabolism and morphogenesis	120,142-143
<i>B. amyloliquefaciens</i> FZB42	NA (plant growth-promoting saprophyte)	Plantazolicin	<i>pzaA-L</i>	narrow-spectrum antibacterial compound	21,150