Streptolysin S-like virulence factors: the continuing *sagA*

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Abstract | Streptolysin S (SLS) is a potent cytolytic toxin and virulence factor that is 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 is just one of an extended family of post-translationally modified virulence factors (the SLS-like peptides) that are produced by some streptococci and other 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).

Impetigo
An acute and highly contagious infection of the surface layers of the skin, characterized by blisters, pustules and yellowish crusts.

Pharyngitis
Inflammation of the pharynx that can be caused by group A, C and G streptococci; also known as strep throat.

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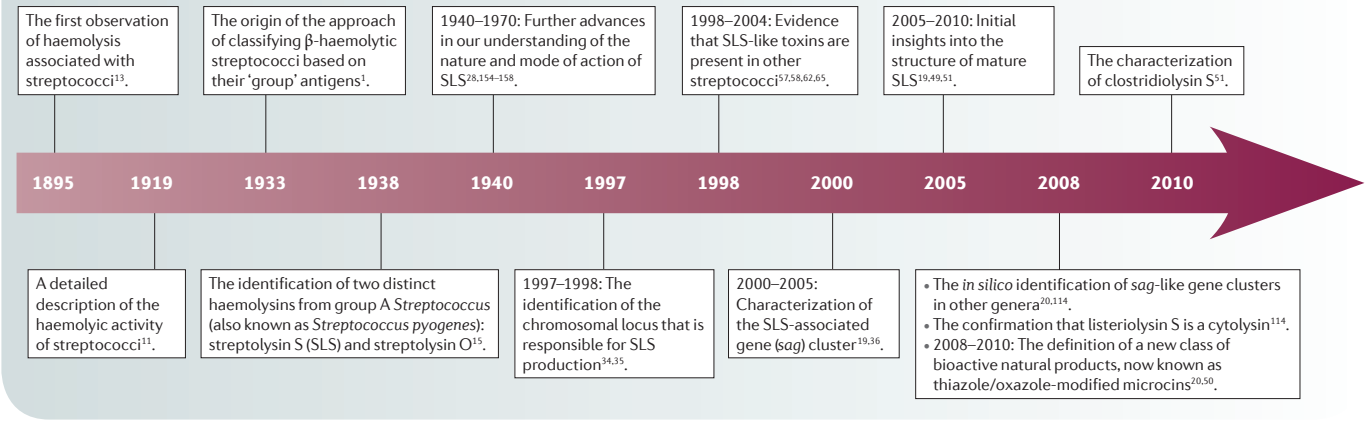
Humans are the natural host and sole reservoir of group A *Streptococcus* (GAS; also known as *Streptococcus pyogenes*)[1,2]. The bacterium can survive and replicate in a variety of body locations, including the skin, throat and blood[3], and is a common cause of illness, especially in children. The 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 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 considerable risk of mortality[6–7], with an estimated 500,000 deaths per year 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 (β-haemolysis)[10–12] was first observed as early as 1895 (REFS 13–14), it was not until 1938 that streptolysin S (SLS) was identified as being one of the two distinct toxins that are responsible for the ability of GAS to lyse mammalian erythrocytes[15]; the other toxin is the unrelated large cholesterol-dependent, oxygen-sensitive streptolysin O (SLO)[16,17]. Although SLS has been subject to rigorous investigation (TIMELINE), isolation of the mature SLS toxin and elucidation of its molecular structure have proved difficult[18].

SLS is a 2.7-kDa peptide that is extensively post-translationally modified before export, resulting in the formation of a distinctive heterocyclic compound[19,20]. In addition to having an unusual structure, SLS is cytolytic only when associated with the bacterial cell surface or in the presence of certain carrier molecules[21–23]. The cytolytic spectrum of the toxin is broad and includes erythrocytes, leukocytes, platelets and subcellular organelles[24–27], but excludes bacteria with intact cell walls[28]. SLS is not immunogenic in the course of natural infection[29], a fact that may reflect its small size and its highly modified nature (which removes proteolytic sites that are critical for antigen digestion and display) as well as, perhaps most importantly, its potent cytotoxicity against cells that are involved in both innate and adaptive immunity[30,31]. Although 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 lysis[32].

It has become apparent that SLS represents the founding member of a class of post-translationally modified virulence peptides, as homologous toxins have now been identified in other *Streptococcus* species. The haemolytic activity of this distinct group of streptococcal toxins is abolished by the addition of trypan blue[33]. Furthermore, gene clusters that are similar to the SLS-associated cluster have been identified in other disease-causing pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus*. In this Review, we describe the identification of SLS, and the GAS chromosomal locus (the SLS-associated gene (*sag*) operon) that encodes both the toxin and the proteins involved in its production, as well as the initial steps towards the

Timeline | **History of streptolysin S research**



characterization of its structure. We provide a brief overview of the role of SLS in virulence and other putative functions. We then discuss the identification of *sag*-like loci in non-GAS streptococci and advances in the identification of similar 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 peptides called the thiazole/oxazole-modified microcins (TOMMs).

The GAS *sag* cluster

The chromosomal locus that is responsible for SLS production was first identified following the characterization of GAS transposon insertion mutants that did not produce SLS[34,35]. The transposons disrupted the promoter for the gene (designated *sagA*) that encodes the 53 amino acid SLS precursor, SagA. Subsequent chromosome-walking studies and genome sequence data identified the contiguous nine-gene *sag* operon (*sagABCDEFGHI*)[36,37] (FIG. 1). Further analysis confirmed that the *sag* operon encodes all the accessory proteins that are required for proper processing and export of SLS[36].

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 class I bacteriocins[36,38]. Like SLS, class I bacteriocins are encoded by an operon that contains a structural gene for a precursor peptide with an amino-terminal 'leader' region and a carboxy-terminal 'core' peptide[39]. The operon also includes genes encoding the machinery that is required for post-translational modification of the core peptide, as well as for cleavage of the leader and export of the mature form of the bacteriocin. In addition to the antimicrobial activity of bacteriocins, in rare cases they also exhibit broader haemolytic and cytolytic properties[40], as is the case for the cytolysin that is produced by *Enterococcus faecalis*[41].

SagA possesses features that are reminiscent of bacteriocin precursor peptides, such as a potential Gly-Gly leader cleavage site that would yield a 23 amino acid leader peptide and a 30 amino acid structural peptide[42,43]. This structural peptide contains an abundance of residues that are frequently the target of post-translational modification in bacteriocins, including Ser, Thr, Cys and Gly[43]. Site-directed mutagenesis of conserved residues in SagA supports its designation as a bacteriocin-like toxin[19], as does the fact that SagA exhibits features that are reminiscent of McbA[36], the precursor of the bacteriocin microcin B17 (MccB17) 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 abundance[36] (FIG. 1a).

The *sagA* gene is followed by *sagBCD*, genes that exhibit low sequence identity to the *mcbBCD* genes that are located in the *E. coli mcb* cluster[20] (FIG. 1b). McbBCD form an enzyme complex — containing a dehydrogenase (McbC), a cyclodehydratase (McbB) and a 'docking' protein (McbD) — that is required for the post-translational conversion of four Ser residues and four Cys residues within McbA into oxazole and thiazole heterocycles, respectively[45–47] (FIG. 2). These modifications are essential for the activity of mature MccB17 (REF. 48). SagB is a 36-kDa species that has some similarity to McbC (22% identity), SagC is a 40.3-kDa protein that is 13% identical to McbB, and SagD is a 51.6-kDa docking protein that is 18% identical to McbD[20,36]. Owing to these similarities, it was hypothesized that SagBCD functions in a similar manner to the McbBCD synthetase complex. It was subsequently established that SagBCD can also catalyse heterocycle formation, as recombinant SagBCD can successfully substitute for McbBCD to process McbA *in vitro*[20].

Further studies with SagBCD established that the complex is indeed responsible for the conversion of SagA into SLS[20]. 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 complex[49]. The modifications involve the conversion, via two distinct steps, of Cys, Ser and Thr residues to thiazole, oxazole and methyloxazole heterocycles, respectively[20] (FIG. 2). SagC removes water from the peptide backbone to produce

Necrotizing fasciitis
A rare but severe type of soft-tissue infection that can be caused by group A *Streptococcus* and 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.

Streptococcal toxic shock syndrome
A rare but extremely severe infection that usually presents in people who have pre-existing skin infections with group A *Streptococcus*. It has a high mortality rate and is characterized by hypotension and shock. Other symptoms can include kidney impairment, abnormality in blood-clotting ability, acute respiratory distress syndrome, rash and local tissue destruction.

β-haemolysis
A phenotype of complete red blood cell lysis (which appears as yellowing and transparency around and under colonies grown on blood agar medium) that is routinely used as a diagnostic tool for the identification of group A *Streptococcus*. It is primarily dependent on streptolysin S, with streptolysin O making a minimal contribution.

thiazoline, oxazoline and methyloxazoline rings, and then a dehydrogenation reaction that is catalysed by SagB formally removes hydrogen to generate aromatic thiazole, oxazole and methyloxazole heterocycles, respectively[20]. SagD is proposed to have a role in the formation of the SagBCD complex and in the regulation of its enzymatic activity[20]. The incorporation of these heterocycles into the unmodified precursor peptide restricts backbone conformational flexibility and provides the mature SLS with a more rigid structure, which is essential for bioactivity, as unstructured peptides would pay too large an entropic penalty to bind to their molecular targets efficiently[50].

Recent research has yielded insights into the structure–activity relationships of SLS, but the precise chemical structure of mature SLS has yet to be fully elucidated. However, site-directed mutagenesis with Ala (to reduce backbone rigidity) and/or Pro (to retain rigidity) suggests that oxazoles are formed at residues Ser34, Ser39, Ser46 and Ser48; a thiazole is formed at Cys32 (REF. 49); and Cys24 and Cys27 are also important residues with respect to haemolytic activity[19,49]. The incorporation of thiazoles, oxazoles or methyloxazoles results in a net loss of 20 Da in peptide mass, and this has been exploited for liquid chromatography–tandem mass spectrometry (LC–MS/MS) with *in vitro*-modified SLS, which has shown that heterocycle formation is catalysed by SagBCD and has confirmed the incorporation of oxazole moieties at Ser46 and Ser48 (REF. 51).

The role of the remaining Sag proteins is less clear. SagF (26.2 kDa) is predicted to be membrane associated, and SagE (25.4 kDa) is expected to be a membrane-spanning peptidase[20,52]. Although it is reasonable to suggest that SagE is the enzyme responsible for leader cleavage, it has also been noted that intact *sagE* is required for viability and that the encoded protein has weak similarity with a candidate bacteriocin immunity (or self-protection) protein, PlnP, of *Lactobacillus plantarum*[19]. SagG (41.7 kDa), SagH (42.2 kDa) and SagI (41.7 kDa) are thought to be membrane proteins that form an ABC-type transporter[19,20] similar to those frequently involved in bacteriocin export[36,53], with SagG displaying the signature ATP-binding-pocket motifs[19].

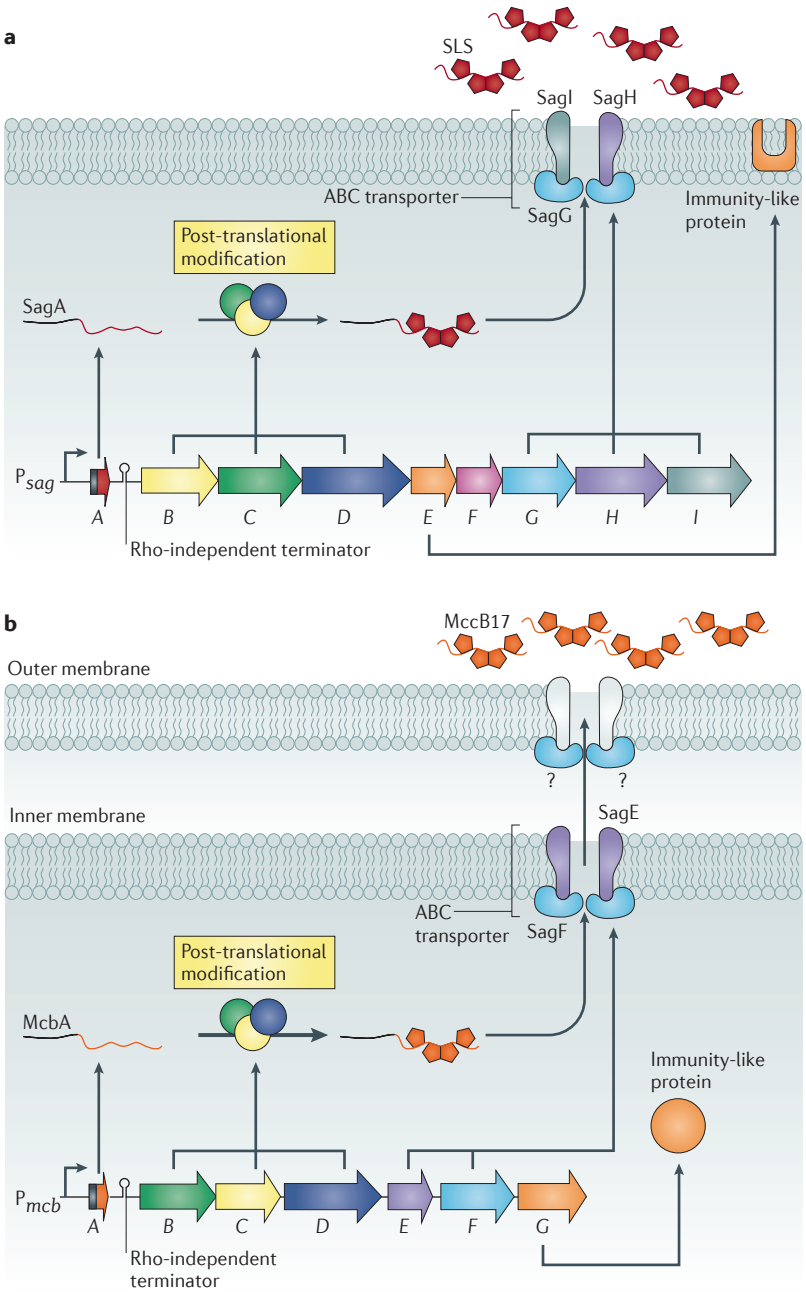


Figure 1 | **Overview of the production, processing and export of streptolysin S and microcin B17.** The carboxy-terminal core peptides of streptolysin (SLS)-associated gene A protein (SagA) (part **a**), which is produced by group A *Streptococcus* (GAS; also known as *Streptococcus pyogenes*), and of microcin B17 protein A (McbA) (part **b**), which is produced by *Escherichia coli*, are post-translationally modified by the SagBCD and McbBCD complexes to form biologically active SLS (a cytotoxin) and microcin B17 (MccB17; a DNA gyrase inhibitor), respectively. The amino-terminal leader sequence (black) is cleaved from the mature core peptide following modification, resulting in a mature peptide product. The 'leaky' Rho-independent terminator sequence between *sagA* and *sagB*, and *mcbA* and *mcbB*, acts as a regulatory mechanism, giving rise to an excess of *sagA* and *mcbA* transcripts compared with the amount of transcripts for the genes encoding the modification and transport machinery.

sag-like loci in other streptococci

The production of SLS-like cytolysins is not exclusively a GAS-associated trait (TABLE 1), as invasive human isolates of the β-haemolytic group C *Streptococcus* (GCS) and group G *Streptococcus* (GGS), which belong to *Streptococcus dysgalactiae* subsp. *equisimilis*, produce SLS-like peptides. Related peptides are also produced by the animal pathogens *Streptococcus iniae* and *Streptococcus equi*.

GCS is a common cause of acute pharyngitis among young adults[54,55]. Acute pharyngitis can also be caused by the normally commensal GGS[56], and GGS strains have been associated with necrotising soft-tissue infections in patients with underlying medical conditions[57]. Both GCS and GGS display a hallmark β-haemolytic phenotype on blood agar[57,58]. The GCS and GGS *sagA* genes are identical, and the corresponding peptides have 89% identity with the GAS SagA[57]. Other conserved features include the *sag* operon promoter, a Rho-independent attenuator located downstream of *sagA*, and eight genes resembling the remaining SLS-associated genes[57].

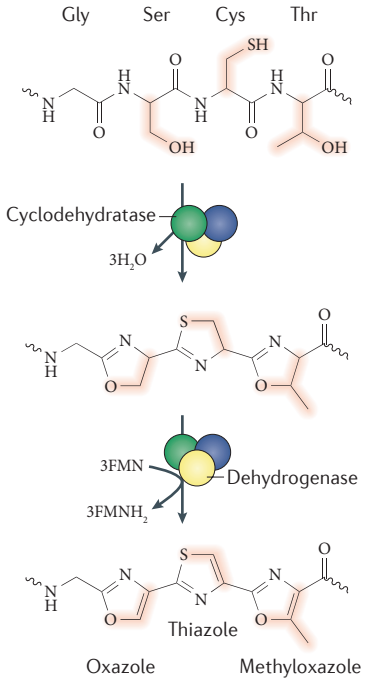


Figure 2 | **Post-translational modification of the streptolysin S precursor.** Streptolysin S (SLS) heterocycles are formed via two distinct steps, catalysed by the combined activity of a zinc tetrathiolate-containing cyclodehydratase (SLS-associated gene C protein (SagC); green) and a dehydrogenase (SagB; yellow) from within a three-protein complex that also contains the docking protein (SagD; blue). SagC removes water from Cys, Ser and Thr residues in the peptide backbone to generate thiazoline, oxazoline and methyloxazoline rings. Subsequently, a flavin mononucleotide (FMN)-dependent dehydrogenation reaction catalysed by SagB removes hydrogen to generate the aromatic thiazole, oxazole and methyloxazole heterocycles. SagD is proposed to have a role in the formation of the SagBCD complex and in the regulation of its enzymatic activity. An example of each heterocyclizable residue is shown for illustrative purposes, with Gly included as a typical residue that is found on the amino-terminal side of the cyclized residues to facilitate the orbital alignment that is required for cyclodehydration[45,50,159,160].

Targeted mutagenesis of GGS *sagA* has been found to abolish β-haemolytic activity, a phenotype that is partially restored on transformation of the mutant with the GAS *sagA* homologue[57].

S. iniae is a β-haemolytic pathogen of commercial fish species and can also be a rare human pathogen[59–61]. Characterization of the genomic region responsible for the haemolytic phenotype of *S. iniae* identified a nine-gene locus that has 73% sequence similarity to the GAS *sag* operon[62]. Furthermore, heterologous expression of *sagA* from *S. iniae* restored haemolytic activity to *sagA*-mutant GAS[63]. Finally, *S. equi* is the causative agent of 'strangles', a prevalent and highly contagious disease of horses[64]. Although 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 an SLS-like toxin[65].

Functions of SLS

A recent screen of clinical GAS samples has shown that 99% of all isolates are haemolytic, whereas the remaining 1% are not predicted to produce SLS and are assumed to be non-haemolytic (Lowry-type) strains[66]. Although such atypical strains have occasionally been associated with human infection[66–68], there is little doubt that SLS has an important role in the pathogenicity of streptococci. Unsurprisingly, the specific mechanisms by which SLS contributes to virulence have been the subject of much investigation (FIG. 3) and include soft-tissue damage, an impact on host phagocytes and a contribution to GAS translocation across the epithelial barrier. SLS also functions as a quorum sensing molecule, and *sagA* mRNA has been implicated in the regulation of other virulence genes. In addition, it has been speculated that SLS contributes to iron acquisition from the host by providing a means through which GAS can access intracellular iron (through the lysis of host red blood cells)[69,70].

Role of SLS in tissue injury. *In vivo* studies have demonstrated that SLS is an important virulence factor in skin and soft-tissue infection, in which it contributes to tissue injury[19]. The virulence of an SLS-negative GAS mutant is substantially reduced in a mouse soft-tissue infection model[35]. 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 that is predicted to interfere with heterocycle formation can render a strain of GAS avirulent in a mouse model of skin infection[49]. The direct toxicity of SLS towards 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 fasciitis[57]. It has been suggested that other virulence factors — such as SLO[19] and the antiphagocytic surface protein M[19,74,75] — as well as host neutrophil-derived oxidants and proteases[23,76–78], interact synergistically with SLS to accelerate necrosis. It has also been established that expression of SLS is required for *S. iniae*-induced local tissue necrosis[62,63].

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 *sag*A mutant did not survive as well as wild-type GAS in killing assays with human whole blood and purified neutrophils[19]. It was subsequently revealed that cell-associated SLS actively destroys neutrophils that are recruited to the site of infection[79]. The elimination of neutrophils (the phagocytic cells that are primarily responsible for the ingestion and killing of GAS) may be a specific virulence mechanism that effectively allows the bacterium to evade the innate immune system[79]. In support of this hypothesis, a paucity or lack of neutrophils in the affected tissues is regarded as an unfavourable prognostic sign in patients suffering from necrotizing fasciitis[80], and a failure of neutrophil influx is also observed in primates that do not survive GAS-associated necrotizing fasciitis and myositis[78]. Macrophages constitute another crucial component of the host's phagocytic defence against GAS infection. GAS can kill macrophages through the

Streptolysin O
(SLO). A thiol-activated, ~57-kDa cytolysin that is produced by group A, C and G streptococci and 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, whereas the oxygen-stable streptolysin S results in a zone of clearing surrounding colonies on the surface of blood agar. SLO is antigenic, resulting in SLO-specific antibodies that are useful for documenting recent exposure to group A *Streptococcus*.

Heterocyclic compound
A compound that has atoms of at least two different elements within its ring structure or structures. With respect to bioorganic chemistry, heterocycles contain one or more carbon atoms and at least one ring member other than carbon.

Carrier molecules
High-molecular-mass molecules, such as non-ionic detergents, albumin, α-lipoprotein, lipoteichoic acid and the RNase-resistant fraction of yeast RNA (RNA core), that can stabilize the haemolytic activity from a streptolysin S (SLS)-producing growing culture or resting cell suspension. SLS is irreversibly inactivated on separation from the carrier or on destruction of the carrier.

Trypan blue
A vital stain that is usually used to selectively colour dead tissue or cells blue. A defining feature of streptolysin S-like peptides is the fact that they are completely inactivated by trypan blue.

Thiazole/oxazole-modified microcins
(TOMMS). A structurally and functionally diverse family of ribosomally produced peptides with post-translationally installed heterocycles derived from Cys, Ser and Thr residues. These modifications rigidify the precursor peptide to endow biological function on the mature natural product.

Table 1 | **Thiazole/oxazole-modified microcins**

Organism	Associated activity	TOMM	Genes	TOMM function	References
Pathogenic bacterial species					
Streptococcus pyogenes	Causes respiratory tract infections, skin diseases and invasive infections	Streptolysin S	*sagABCDEFGHI*	Haemolytic exotoxin	15,34,36
Streptococcus dysgalactiae subsp. *equisimilis*	Causes pharyngitis and invasive infections	Streptolysin S	*sagABCDEFGHI*	Haemolytic exotoxin	57
Streptococcus iniae	A fish pathogen and rare human pathogen that causes invasive infections	Streptolysin S	*sagABCDEFGHI*	Haemolytic exotoxin	62
Streptococcus equi	A horse pathogen	Streptolysin S	ND	Haemolytic exotoxin	65
Listeria monocytogenes	Causes gastroenteritis, septicaemia and meningitis	Listeriolysin S	*llsAGHXBYDP*	Haemolytic exotoxin	20,114
Clostridium botulinum and *Clostridium sporogenes*	Causes botulism poisoning	Clostridiolysin S	*closABCDEFGHI*	Haemolytic exotoxin	20,51,114
Staphylococcus aureus str. RF122	Causes bovine mastisis	Staphylysin S	*stsAGHBB′CC′DP*	Haemolytic exotoxin	20,49,114
Non-pathogenic bacterial species					
Escherichia coli	A gut commensal with pathogenic potential	Microcin B17	*mcbABCDEFG*	Antibacterial (DNA gyrase inhibitor)	44,46,47
Prochloron sp.	A photosynthetic endosymbiont of the ascidian *Lissoclinum patella*	Patellamide D	ND	Reverses multidrug resistance in a human leukaemia cell line	148,149
Streptomyces sp. TP-A0584	A soil inhabitant	Goadsporin	*godABCDEFGHRI*	Antibiotic, and promoter of secondary metabolism and morphogenesis	123,143,144
Bacillus amyloliquefaciens str. FZB42	A saprophyte that promotes plant growth	Plantazolicin	*pznFKGHIAJCDBEL*	Narrow-spectrum antibacterial	20,151

mcb, microcin B17 gene; ND, not determined; *sag*, streptolysin S-associated gene; TOMM, thiazole/oxazole-modified microcin.

SLS- and SLO-mediated activation of an inflammatory programmed cell death pathway[81]. 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 other factors may also have a prominent role.

GAS is known to produce virulence factors that diminish the host's immune response to infection[82–84]. SLS is thought to contribute to this by affecting the ability of the host cell to produce signals that are chemotactic for neutrophils[85]. Using a zebrafish model, it was shown that an SLS mutant was significantly less virulent than wild-type GAS and was associated with a more robust recruitment of neutrophils[85].

Finally, little is known about the entry and subsequent multiplication of *S. equi* following the exposure of a susceptible equine host[86], but data indicate that virulence is the result of a potent antiphagocytic effect and the failure of innate immune defences[87], suggesting that the SLS-like toxin of this bacterium may play an important part.

Role of SLS in GAS translocation. Systemic dissemination of GAS involves bacterial colonization of the pharynx or damaged skin, followed by penetration of the epithelial barrier. The mechanisms underlying the adherence of GAS to epithelial cells and its subsequent internalization have been extensively studied[88–91]. Most internalized GAS cells are eliminated by intracellular killing. However, it has been speculated that the programmed cell death induced in epithelial cells by massive adherence or internalization of GAS reduces the barrier function of the epithelium, providing GAS with access to deeper tissues through intracellular invasion[92–94]. Paracellular invasion by GAS has also been noted[95] and, notably, recent research has identified SLS as a critical factor in this process[96]. SLS recruits the host Cys protease calpain to the plasma membrane by an as-yet-uncharacterized mechanism and then uses the proteolytic activity of this enzyme to degrade intercellular junctions and thus allow invasion through a paracellular route[96].

SLS as a signalling molecule. Many species of bacteria use a cell density-dependent response system, or quorum sensing, to regulate gene expression[97], and several GAS virulence genes are modulated by quorum sensing[98,99]. *luxS* (which encodes *S*-ribosylhomocysteine lyase) is essential for the production of autoinducer 2 (AI2) of the AI2 quorum sensing pathway in a diverse range of bacterial species[100], and GAS strains with mutations in the *luxS* homologue display a number of altered phenotypes[101]. Notably, the SLS activity of a *luxS* mutant was found to be enhanced as a result of increased *sagA* transcription[101].

Chromosome-walking studies
Studies using a technique to identify and characterize regions of DNA by the sequential isolation of overlapping DNA sequences, starting with a known fragment of DNA.

Bacteriocin
A small ribosomally synthesized, heat-stable peptide that is produced by one bacterium and is active against other, either in the same species (narrow spectrum) or across genera (broad spectrum).

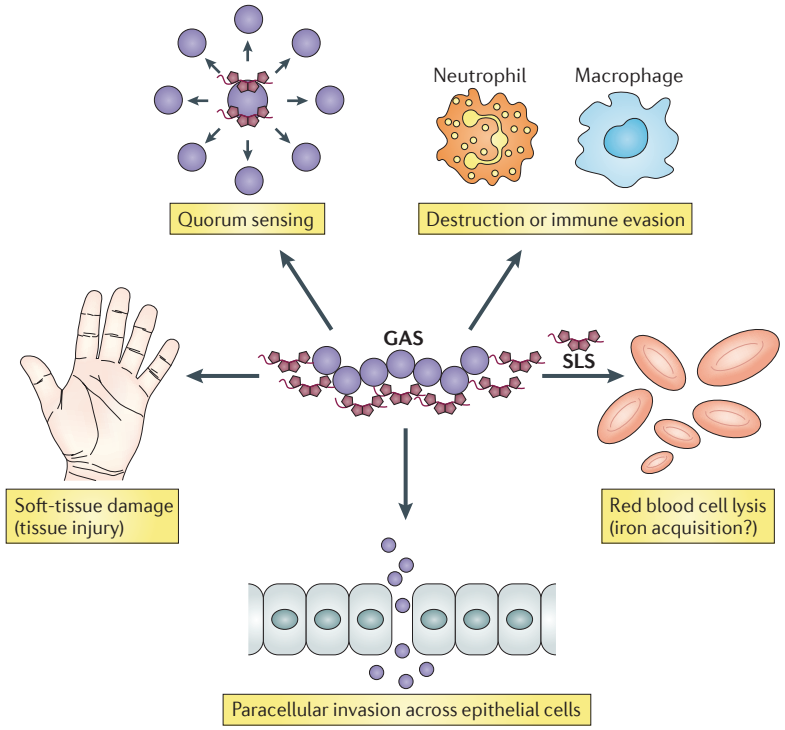


Figure 3 | **Summary of the functions of streptolysin S.** The mechanisms by which streptolysin S (SLS) is known to contribute to the virulence of group A *Streptococcus* (GAS; also known as *Streptococcus pyogenes*) include soft-tissue damage, an impact on host phagocytes and a contribution to the paracellular translocation of GAS. In addition, SLS-associated gene A (*sagA*) mRNA and the associated 'pleiotropic effects locus' (*pel*) mRNA affect virulence through their impact on the expression of other virulence genes. SLS also functions as a signalling molecule, and it has been proposed to contribute to iron acquisition from the host.

Indeed, SLS expression increases with cell density[35], and it was initially suspected that SLS itself might act as a quorum sensing molecule[102]. Several bacteriocins are known to be regulated by quorum sensing[38], and in some instances (for example, the lantibiotic nisin) the structural peptides also function as signalling molecules and induce their own expression on activation of the density-dependent autoinduction loop[103]. Importantly, it has since been established that expression of *sagA* is upregulated by exposure to SLS[102]. It should also be noted that the expression of *sagA* is under transcriptional control of the GAS global regulators that are encoded by *covR* (also known as *csrR*) and *covS* (also known as *csrS*), *mga*, *rofA*, the *fas* operon and *nra*[104–110].

Finally, global regulatory functions have been attributed to an untranslated mRNA, designated *pel* for 'pleiotropic effects locus', that matches the *sagA* gene[111,112]. It has been suggested that *sagA* mRNA is involved in the pre- and post-translational control of other GAS virulence factors, including M proteins, the capsule protein streptopain (SpeB) and streptokinase[111–113]. In contrast to these results, it has been found by others that elimination of *sagA* does not produce significant pleiotrophic effects[19], suggesting that the role of *sagA* and/or *pel* mRNA in the regulation of other virulence factors may differ in a strain-specific manner.

Class I bacteriocins
Antimicrobial peptides that are extensively post-translationally modified in their active form, including the lantibiotic (lanthionine-containing) family of bacteriocins.

Microcin B17
(MccB17). A class I bacteriocin that is produced by strains of *Escherichia coli* and is active against closely related bacterial species, targeting the essential enzyme DNA gyrase.

Lowry-type
A designation for atypical, completely non-haemolytic group A *Streptococcus* strains, named after the initial isolation of such a strain (by James and McFarland in 1971) from an outbreak of rheumatic fever at Lowry Air Force Base, Colorado, USA.

sag-like loci in other bacterial genera

Until recently, post-translationally modified virulence peptides had rarely been reported and SLS-like cytolysins had been exclusively associated with the genus *Streptococcus*. As noted above, however, many similarities exist between the GAS *sag* operon and the MccB17 biosynthesis operon that is present in some *E. coli*. This observation prompted a search of public genomic databases, in the belief that other bacteria would use related machinery to introduce Ser-, Thr- and Cys-derived heterocycles into a wider variety of ribosomally produced peptides[20,114]. 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*[20,114] (FIG. 4; TABLE 1). In addition, more distantly related clusters were identified in an even more diverse collection of bacteria[20]. This led to the definition of a new class of compounds that are characterized by a biosynthetic gene cluster encoding a small precursor peptide and three adjacent synthetase proteins that serve to introduce thiazole, oxazole and methyloxazole heterocycles onto the ribosomally produced protoxin scaffold. These bioactive natural products and their biosynthetic gene clusters are now referred to as TOMMs[20,50]. Although the biological purposes of the majority of newly identified TOMMs have not been uncovered, it is likely, based on their similarity to McbA and SagA, that some will act as either DNA gyrase inhibitors or membrane-damaging agents[115].

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 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 hypervariable nature[114,116]. Furthermore, although short ORFs encoding proteins of 50–70 residues that are rich in Cys, Ser and Thr are usually found in organisms with *sagBCD*-like genes as adjacent ORFs[20,114], TOMM peptides are sometimes situated far from the genes encoding the thiazole-, oxazole- and methyloxazole-forming machinery, and in such cases it can be difficult to assign the substrate for a particular TOMM pathway[117]. The situation is further complicated by the observation that numerous substitutions in the C-terminal core sequence of the propeptide can often be tolerated[49]. This can result in the production of many similar peptide products[50,118–120] by 'natural combinatorial biosynthesis'. In addition, new families of more unusual modifying enzymes are often not sufficiently related to characterized relatives to be identified by BLAST (basic local alignment search tool) searching[20,121,122].

Several factors can increase confidence in the designation of an ORF as encoding a TOMM precursor, including the predicted protein having sequence similarity to previously identified TOMM precursors, having a suitable leader peptide cleavage motif and having a C-terminal core region that is rich in heterocyclizable

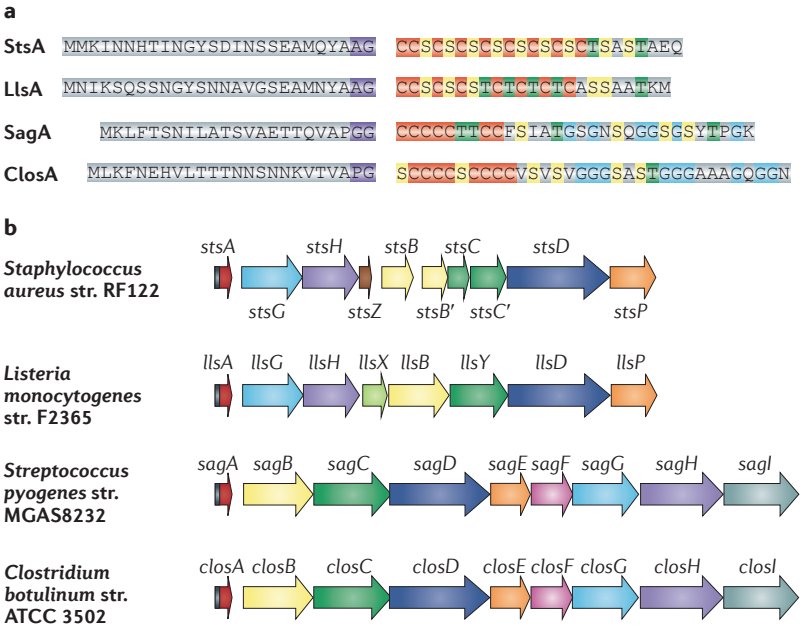


Figure 4 | **Amino acid sequence and operon structure of selected thiazole/oxazole-modified microcins (TOMMs). a** | The amino acid sequence of the unmodified stapholysin S precursor (StsA), listeriolysin S precursor (LlsA), streptolysin S precursor (streptolysin S-associated gene A protein (SagA)) and clostridolysin precursor (ClosA) from *Staphylococcus aureus*, *Listeria monocytogenes*, group A *Streptococcus* (GAS; also known as *Streptococcus pyogenes*) and *Clostridium botulinum*, respectively. The predicted leader regions are to the left and terminate in putative leader cleavage sites (purple). Residues that are potentially involved in the modification of the precursor peptides are indicated in orange (Cys), yellow (Ser), green (Thr) and blue (Gly). Amino acids that cannot be modified are shown in grey. 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 streptolysin S (SLS) is an assemblage of modified SagA peptides). **b** | *sag*-like gene clusters in *S. aureus*, *L. monocytogenes*, *S. pyogenes* and *C. botulinum* strains. Related genes are indicated by colour. In each case, the respective gene designations are: *A*, structural gene; *B*, dehydrogenase; *C* and *Y*, cyclodehydratase; *D*, docking protein; *E* and *P*, CAAX protease; *G*, *H* and *I*, ABC-type transporter components; *Z*, *X* and *F*, proteins of unknown function. In the case of *S. aureus* str. RF122, the genomic map appears to be fragmented, giving multiple ORFs for several biosynthetic proteins.

residues in the predicted protein[20,49] (FIG. 4). The detection of genes encoding enzymes involved in thiazole and oxazole synthesis, dehydroalanine production[123] and peptide macrocyclization[116,118,122,124] provides further support when annotating gene clusters that are responsible for the biosynthesis of post-translationally modified peptides. The identification of a TOMM biosynthesis cluster is also facilitated by the tendency of the modification enzymes to cluster with other genes that are associated with the cleavage and export of the final product[20,36,46].

It is clear that the extent of structural variation in and the biological impact of TOMMs 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[50,117], will be applied to uncover additional members of this family of modified peptides, as well as to help identify cognate modification and export genes.

Quorum sensing
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.

Myositis
A general term for inflammation of the skeletal muscles.

Paracellular invasion
The translocation of pathogens across an epithelial barrier by passing between the host cells.

***Listeriolysin S of* L. monocytogenes.** *L. monocytogenes* is an intracellular pathogen that is usually transmitted to humans through contaminated food products[125]. It has a high mortality rate in pregnant women, neonates and individuals with a compromised immune system[126]. Strains of *L. monocytogenes* are divided into three evolutionary lineages[127], 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 that are associated with this often fatal pathogen[128,129].

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 to be produced by *L. monocytogenes*[130,131]. 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 cluster[114]. The LLS structural gene within this cluster, *llsA*, encodes a peptide consisting of an N-terminal leader region, and a C-terminal core region with an extreme predominance of Cys, Ser and Thr residues, as well as a putative Ala-Gly leader cleavage motif[114]. As the predicted *llsA* promoter 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 an SLS-like cytolysin[114]. Like SLS, LLS was found to be active in a cell-associated form but inactive in cell-free situations in the absence of a stabilizer[114]. It is now apparent that previous detection of LLS-mediated 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 the *llsA* promoter 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 that is necessary for the production of mature, active LLS[114,132]. LLS is believed to be post-translationally modified in a similar manner to SLS, a theory that is supported by the fact that a SagA–LlsA chimaera (the SagA leader sequence fused to the LlsA core peptide) is converted into a cytolytic entity by SagBCD *in vitro*[49]. Other proteins encoded by the operon include LlsG and LlsH, which are 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[114,132,133]. The gene *llsX* is not homologous to any known gene and thus can be used to identify LLS-positive strains of *L. monocytogenes* by real-time PCR[132]. Deletion mutagenesis has established that six of the seven genes (*llsGHXBYDP*) that are located downstream of *llsA* in the *lls* operon are essential for LLS activity, with only *llsP* being non-essential[114,132].

LLS contributes to pathogenesis, as evidenced by the significantly reduced levels of an LLS-negative mutant in the liver and spleen of mice following intraperitoneal inoculation, compared with levels of the corresponding wild-type bacteria[114]. Furthermore, wild-type *L. monocytogenes* survives significantly better than the

LLS-negative mutant in purified human polymorphonuclear neutrophils. Notably, polymorphonuclear neutrophils are crucial for the resolution of *L. monocytogenes* infections[134]. Given the oxidative stress-inducible nature of the *lls* promoter and the contribution of LLS to neutrophil endurance, it has been suggested that LLS contributes to virulence by enhancing the survival of bacteria that are still retained in the phagosome upon phagosome–lysosome fusion[114]. The LLS-encoding TOMM cluster is also known as *Listeria* pathogenicity island 3 (LIPI-3)[114]. Given that LIPI-3 is consistently absent from all lineage II and III *L. monocytogenes* strains, and that few of the lineage I strains that are associated with outbreaks lack LIPI-3, it has been postulated that this gene cluster is the long-sought-after explanation for the enhanced virulence of a proportion of lineage I *L. monocytogenes* strains[114].

Stapholysin S of* Staphylococcus aureus *str. RF122. A *sag*-like cluster is also present in the genome of *S. aureus* str. RF122 (REFS 20,114), a pathogenic strain that is responsible for bovine mastitis[135]. Of the known *sag*-like clusters, this *S. aureus* cluster is most closely related to the *lls* cluster[20,49,114]. The putative structural gene again encodes a peptide consisting of a proposed N-terminal leader region, a C-terminal precursor peptide with an abundance of Cys, Ser and Thr residues, and an Ala-Gly suspected leader cleavage site[20,114]. Thus, it was predicted that mature 'stapholysin S' would act as a cytolysin[20]. This hypothesis was experimentally validated when a chimeric substrate comprising the leader peptide of SagA fused to the C terminus of stapholysin S was converted into a cytolytic entity by SagBCD *in vitro*[49].

Clostridiolysin S of clostridia. Using comparative genomic analysis, nine-gene *sag*-like clusters were identified in the genomes of clostridia, including the biological warfare-associated pathogen *C. botulinum* and the food pathogen *Clostridium sporogenes*[51]. *C. botulinum* is an anaerobic, spore-forming, rod-shaped bacterium that causes the potentially fatal neuroparalytic diseases foodborne, infant, wound and inhalation botulism, as well as other invasive infections, through the elaboration of potent neurotoxins[136]. *C. sporogenes* is the *C. botulinum* counterpart that does not produce botulinum toxin[137].

Before the functionality of the *C. botulinum sag*-like cluster had been confirmed, *in vitro* production of the bioactive natural product via SagBCD modification supported its designation as a TOMM, as did the haemolytic nature of the biotoxin that was generated[20]. This is not unexpected in light of the gene organization in the *C. botulinum* cluster (*closABCDEFGHI*) and the amino acid sequences of the encoded proteins, which correspond closely to the organization and encoded proteins of the *sagABCDEFGHI* cluster. In fact, *C. botulinum* harbours the most closely related *sag*-like genetic cluster known outside of the streptococci[49]. The cytolytic gene product of *closA* has been named clostridiolysin S, or CLS[51]. The functional equivalence of the clostridial *sag*-like gene clusters to the SLS biosynthesis pathway genes was confirmed by the complementation of targeted GAS *sag* operon knockouts with the corresponding clostridial genes[51]. The availability of the complete genome sequence of *C. sporogenes*[138] allowed this safer organism to be used for mutagenesis studies, which established that the *clos* cluster is indeed responsible for a haemolytic phenotype[51].

A more in-depth investigation of the *clos* genes further highlighted their 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 biotoxin[51]. ClosG, ClosH and ClosI are ABC transporters and therefore probably export the mature haemolytic product. ClosE has been annotated as an immunity protein but, like SagE, is similar to the CAAX protease superfamily[133] and so might be responsible for leader cleavage from the post-translationally modified propeptide[51]. ClosF is a protein of unknown function.

The *in vitro* reconstitution of CLS activity allowed a direct confirmation by mass spectrometry that the nontoxic precursor, ClosA, is post-translationally modified by the synthetase enzymes to contain heterocyclic moieties[51]. A heterocycle was identified at position Thr46 within ClosA, and the dramatically negative impact of a Thr46Ala substitution on haemolytic activity confirmed the importance of heterocyclic conversion at this site[51]. 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 that are discussed above, similar TOMM-associated clusters are widely disseminated in the genomes of a diverse group of microorganisms spanning six phyla[20]. Indeed, even archaea such as the thermophile *Pyrococcus furiosus*, can contain heterocycle-forming synthetases[20]. In addition to the SLS-like toxins described above, which have established or suspected roles in virulence, there are several other TOMM biosynthesis clusters of note (TABLE 1). As a consequence of the recent TOMM family expansion, this family now encompasses such diverse bacterial products as the microcins[139], thiazolyl peptides[140,141] and cyanobactins[142]. Emerging TOMM subfamilies include putative thiazole-containing heterocyclic bacteriocins associated with the genus *Bacillus*[117], and a group of nitrile hydratase and Nif11-related precursor peptides[50].

A gene cluster that has an identical arrangement to the *mcb* cluster has been found in the plant symbiont *Pseudomonas putida* str. KT2440 (REF. 139), and it is speculated that, like MccB17, the product of this gene cluster functions as an inhibitor of DNA gyrase[20]. Many other bacteria harbour a *sag*-like gene cluster that is not associated with the production of a cytolysin, such as the goadsporin-producing microorganism, *Streptomyces* sp. TP-A0584 (REF. 143). 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[115,123,144]. Other diverse functions encoded by TOMM peptides include the inhibition of

Natural combinatorial biosynthesis
The production of a library of related compounds by an organism. In the case of some thiazole/oxazole-modified microcin biosynthesis pathways, a single enzyme complex processes numerous substrates that have a common recognition motif but variable structural carboxyl termini. One striking example is the cyanobactin family of natural products.

ribosome-mediated protein synthesis[145] by the thiazolyl peptides, a family of >50 bactericidal antibiotics[146,147], and the reversal of multidrug resistance in a human leukaemia cell line by the cyanobactin patellamide D[148,149].

Last, the genome of *Bacillus amyloliquefaciens* str. FZB42, a Gram-positive saprophyte that promotes plant growth and can suppress the growth of bacterial and fungal pathogens of plants[150], was recently shown to contain a novel TOMM cluster[20]. Subsequent investigations have revealed that this unique TOMM, plantazolicin, functions as a narrow-spectrum antibacterial compound[151]. It has been suggested that the biological role of this natural product is to suppress the growth of closely related competitors within the plant rhizosphere[151].

It is apparent that these ribosomally produced natural peptides represent a largely hidden arsenal of active small molecules that, although grouped as a consequence of their 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 the biosynthetic genes that are 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 suspected[152] and can contribute substantially to the virulence potential of pathogenic bacteria. Of the myriad putative TOMM-encoding clusters that have been identified, those associated with *L. monocytogenes*, *C. botulinum*, C. *sporogenes* and *S. aureus* are particularly noteworthy as a consequence of their similarity to the *sag* cluster of GAS 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, suggest 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 beneficial as adjuncts to surgical and antibiotic management of human streptococcal infection. The observation that synthetic peptides corresponding to the SLS precursor retain sufficient features of the mature toxin to raise neutralizing antibodies[32,153] suggests that subunits or inactivated toxoids of each haemolysin could have substantial immunogenicity. Insights gained through the *in vitro* application of the SagBCD machinery and the SagA peptide[49] could also be used to guide the design of artificial toxins with a view to structure-based vaccines. Furthermore, because the targeting of 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 appreciated[50]. The proven success of *in silico* analyses suggests that further studies of this type will help uncover the biosynthetic systems for novel TOMM (and other natural product) secondary metabolites and will aid in the definition of the evolutionary routes and interrelationships of the rapidly expanding TOMM ribosomal-peptide group. Such studies inform structural investigations into natural products and pave the way for combinatorial-biosynthesis studies and rational engineering.

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