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# Identification of the minimal cytolytic unit for streptolysin S and an expansion of the toxin family

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## Abstract

**Background:** Streptolysin S (SLS) is a cytolytic virulence factor produced by the human pathogen *Streptococcus pyogenes* and other *Streptococcus* species. Related “SLS-like” toxins have been characterized in select strains of *Clostridium* and *Listeria*, with homologous clusters bioinformatically identified in a variety of other species. SLS is a member of the thiazole/oxazole-modified microcin (TOMM) family of natural products. The structure of SLS has yet to be deciphered and many questions remain regarding its structure-activity relationships.

**Results:** In this work, we assessed the hemolytic activity of a series of C-terminally truncated SLS peptides expressed in SLS-deficient *S. pyogenes*. Our data indicate that while the N-terminal poly-heterocyclizable (NPH) region of SLS substantially contributes to its bioactivity, the variable C-terminal region of the toxin is largely dispensable. Through genome mining we identified additional SLS-like clusters in diverse *Firmicutes*, *Spirochaetes* and *Actinobacteria*. Among the *Spirochaete* clusters, naturally truncated SLS-like precursors were found in the genomes of three Lyme disease-causing *Borrelia burgdorferi* sensu lato (*Bbsl*) strains. Although unable to restore hemolysis in SLS-deficient *S. pyogenes*, a *Bbsl* SLS-like precursor peptide was converted to a cytolytin using purified SLS biosynthetic enzymes. A PCR-based screen demonstrated that SLS-like clusters are substantially more prevalent in *Bbsl* than inferred from publicly available genome sequences.

**Conclusions:** The mutagenesis data described herein indicate that the minimal cytolytic unit of SLS encompasses the NPH region of the core peptide. Interestingly, this region is found in all characterized TOMM cytolytins, as well as the novel putative TOMM cytolytins we discovered. We propose that this conserved region represents the defining feature of the SLS-like TOMM family. We demonstrate the cytolytic potential of a *Bbsl* SLS-like precursor peptide, which has a core region of similar length to the SLS minimal cytolytic unit, when modified with purified SLS biosynthetic enzymes. As such, we speculate that some *Borrelia* have the potential to produce a TOMM cytolytin, although the biological significance of this finding remains to be determined. In addition to providing new insight into the structure-activity relationships of SLS, this study greatly expands the cytolytin group of TOMMs.

**Keywords:** Streptolysin S, *Streptococcus pyogenes*, Group A *Streptococcus*, Thiazole/oxazole-modified microcin, Cytolytin, *Borrelia burgdorferi* sensu lato, Lyme disease, Linear azole-containing peptide

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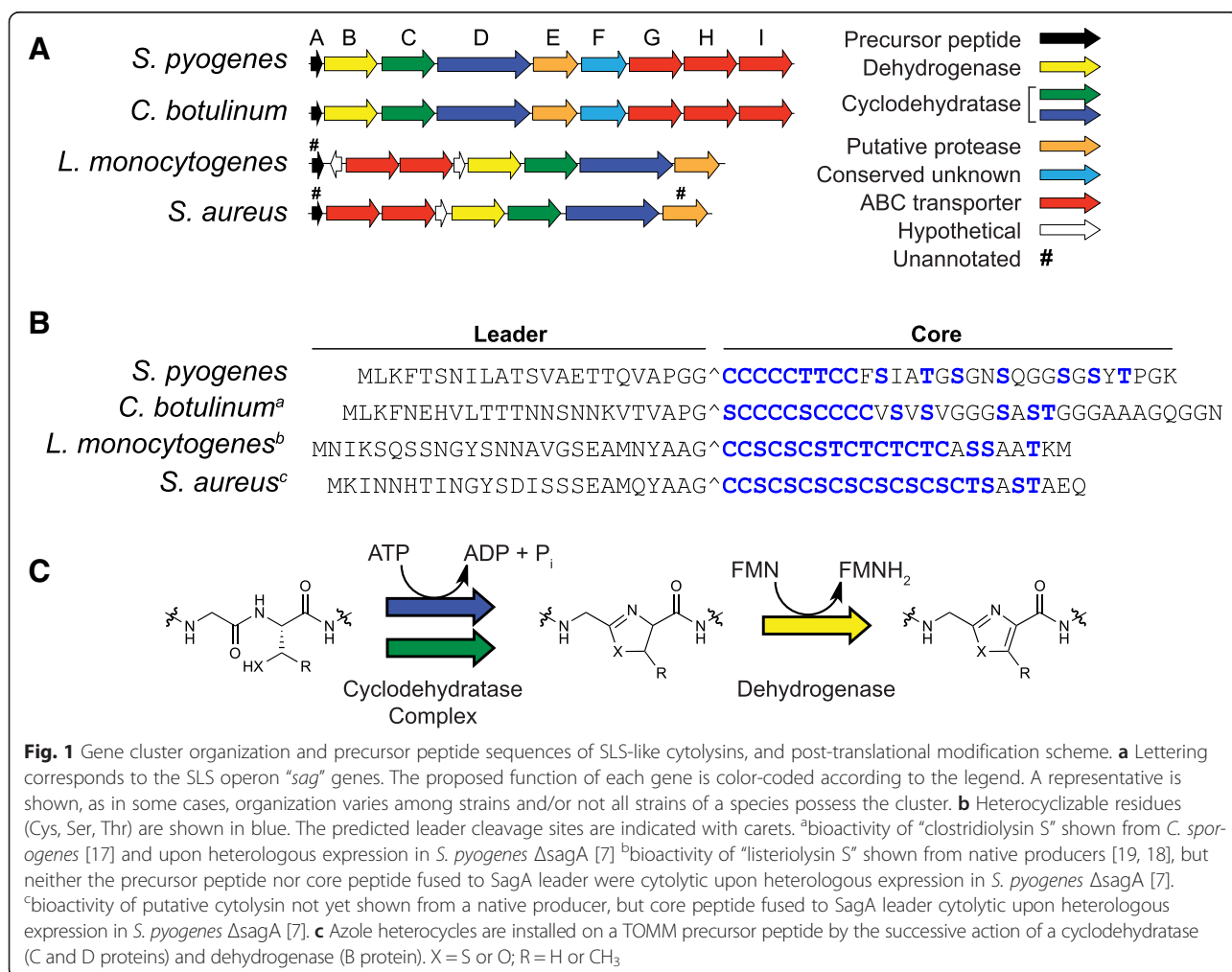
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## Background

*Streptococcus pyogenes* (Group A *Streptococcus*) commonly causes mild diseases such as pharyngitis and impetigo, and in rare cases, invasive infections such as necrotizing fasciitis and streptococcal toxic shock syndrome [1]. While the characteristic  $\beta$ -hemolytic phenotype of *S. pyogenes* grown on blood agar has long been known [2], more than four decades passed before the responsible hemolytic/cytolytic factor was identified [3], streptolysin S (SLS) [4]. The eventual discovery of the SLS biosynthetic gene cluster [5] prompted investigations that subsequently designated SLS a member of the thiazole/oxazole-modified microcin (TOMM) group of natural products [6–8]. TOMMs are a class of functionally and structurally diverse ribosomal peptides that are posttranslationally modified to contain the eponymous thiazole and (methyl)oxazole heterocycles derived from select cysteine, serine, and threonine residues [8]. The SLS biosynthetic operon (Fig. 1a) encodes a precursor peptide, SagA (Fig. 1b), and three heterocycle-forming proteins, SagBCD [5, 7]. Similar to other characterized

ribosomal natural products, the SagA N-terminal leader peptide contains residues recognized by the modifying enzymes, while the C-terminal core peptide undergoes heterocyclization [7, 9, 10] (Fig. 1b and c). The suspected protease, SagE, is believed to remove the leader peptide [11, 12], permitting the export of mature and bioactive SLS via a dedicated ABC transporter, SagGHI (Fig. 1a).

SLS is the founding member of a group of homologous TOMM cytolytins that have been characterized in other *Streptococcus* species (Additional file 1: Figure S1A), including invasive human isolates of the  $\beta$ -hemolytic Group C and Group G streptococci, which belong to *S. dysgalactiae* subsp. *equisimilis* [13]. SLS variants are also produced by the animal pathogens *S. iniae* [14] and *S. equi* [15]. More recently, *S. anginosus*, a human commensal organism and emerging opportunistic pathogen, has been shown to produce twin SLS homologs [16]. Notably, SLS-like toxins are not limited to *Streptococcus*, being produced by other *Firmicutes* including select strains of *Clostridium* [17] and *Listeria* [18–20] (Fig. 1a and b).



Despite intense study, the precise mechanism of SLS toxicity towards mammalian cells remains incompletely understood [21]. SLS has also thus far been recalcitrant to structural elucidation owing to its poor physicochemical properties, although an extensive mass spectral analysis detected two oxazole moieties at positions S46 and S48 of SLS modified with purified biosynthetic enzymes [17] (Fig. 2a). Previous mutagenesis studies indicate that residues spanning the entire SLS core peptide are required for *S. pyogenes* hemolysis [7, 22] (Additional file 1: Figure S1B). This contrasts with the highly conserved N-terminal poly-heterocyclizable (NPH) region and variable C-terminus of SLS natural variants (Additional file 1: Figure S1A). Here, we sought to reconcile these discrepancies by probing the contribution of the C-terminus to SLS bioactivity. We demonstrate that severely C-terminally truncated SLS peptides expressed in a *sagA* deletion mutant of *S. pyogenes* M1 5448 (*S. pyogenes*  $\Delta$ *sagA*) retain hemolytic activity and posit that the NPH region found in all TOMM cytolysins is the defining feature of the family (Fig. 2a, underlined portion). Using bioinformatics, we identified a set of SagA homologs containing the NPH region in TOMM biosynthetic clusters in additional *Firmicutes*, as well as the *Actinobacteria* and *Spirochaete* phyla. We demonstrate that a naturally truncated precursor peptide encoded by a member of *Borrelia burgdorferi* sensu lato (*Bbsl*) species group can be matured into a cytolysin using purified SLS biosynthetic enzymes. Notably, several *Bbsl* cause Lyme disease, the most commonly reported tick-borne illness in the Northern hemisphere [23, 24],

and our PCR-based screen revealed that genes encoding SLS-like precursors are prevalent in diverse *Bbsl* isolates. While our preliminary findings are intriguing, it remains to be seen if these putative cytolytic TOMMs are produced by *Borrelia*, and if so, whether they function to enhance virulence. Together, our results identify the minimal cytolytic determinant of SLS and indicate that there are potentially considerably more producers of related cytolysins than currently appreciated.

## Results and discussion

### Expression of truncated SLS precursors in *S. pyogenes* $\Delta$ *sagA* reveals the importance of N-terminal heterocyclizable region for hemolytic activity

An alignment of experimentally validated SLS core peptides revealed a highly conserved N-terminal region, containing 9–10 contiguous heterocyclizable residues, while the C-terminus is considerably more variable in terms of both identity of residues and length (Additional file 1: Figure S1A). Since only the NPH region of the core peptide is apparently under strict evolutionary pressure, we postulated that the C-terminal region might be less important for SLS function. Indeed, heterologous expression data support this, as peptides with divergent C-termini were able to restore the  $\beta$ -hemolytic phenotype in *S. pyogenes*  $\Delta$ *sagA* [7] (Fig. 1b). To test our hypothesis, we installed premature stop codons in *sagA* (Fig. 2a) and expressed the resultant C-terminally truncated SLS derivatives in *S. pyogenes*  $\Delta$ *sagA*. Unfortunately, the ring-state of processed SagA cannot be



**Fig. 2** Assessment of the hemolytic activity of truncated SLS precursor peptides expressed in *S. pyogenes*  $\Delta$ *sagA*. **a** Residues of the SagA core peptide are numbered. Potentially modified residues of SLS are blue, while those known to be heterocyclized when SagA is modified with purified SagBCD are purple. The predicted leader cleavage site is indicated with a caret. The positions of stop codons introduced in SagA are marked with asterisks (yields truncation mutants listed in panel B). The minimal core region required for hemolytic activity of SLS in *S. pyogenes*  $\Delta$ *sagA* (SagA<sup>24-34</sup>) is underlined (see panel B). **b** The labels correspond to the SagA truncation mutants described in panel A. A representative colony on blood agar of GAS M1  $\Delta$ *sagA* expressing each precursor from pDCerm is shown. The colony labeled “Empty” was a negative control containing pDCerm vector

readily determined, even with advanced spectroscopic methods [17, 6]. We thus relied on a simple phenotypic assay, i.e.  $\beta$ -hemolysis on blood agar, to simultaneously evaluate successful production and export of SLS derivatives.

Consistent with the divergence of the core peptides (Additional file 1: Figure S1A), SLS retained significant activity upon C-terminal truncation. In fact, *S. pyogenes*  $\Delta$ sagA expressing SLS derivatives missing up to 17 residues (SagA<sup>1–36</sup>) showed comparable levels of  $\beta$ -hemolysis on blood agar to that complemented with full-length SagA (Fig. 2b). Hemolytic activity substantially decreased with additional truncation to yield SagA<sup>1–35</sup> and SagA<sup>1–34</sup>, with activity being undetectable in the case of SagA<sup>1–33</sup> (Fig. 2b). These findings allowed us to define SagA<sup>24–34</sup> as the minimal unit required for hemolytic activity of SLS expressed by *S. pyogenes*, assuming leader cleavage at the predicted site (Fig. 2a). Interestingly, this segment encompasses the entirety of the conserved NPH region (Additional file 1: Figure S1A), as well as just two additional residues (Fig. 2a). Based on the predicted leader peptide cleavage site [22], this indicates that only 11 core residues are required for detectable bioactivity of SLS expressed by *S. pyogenes*.

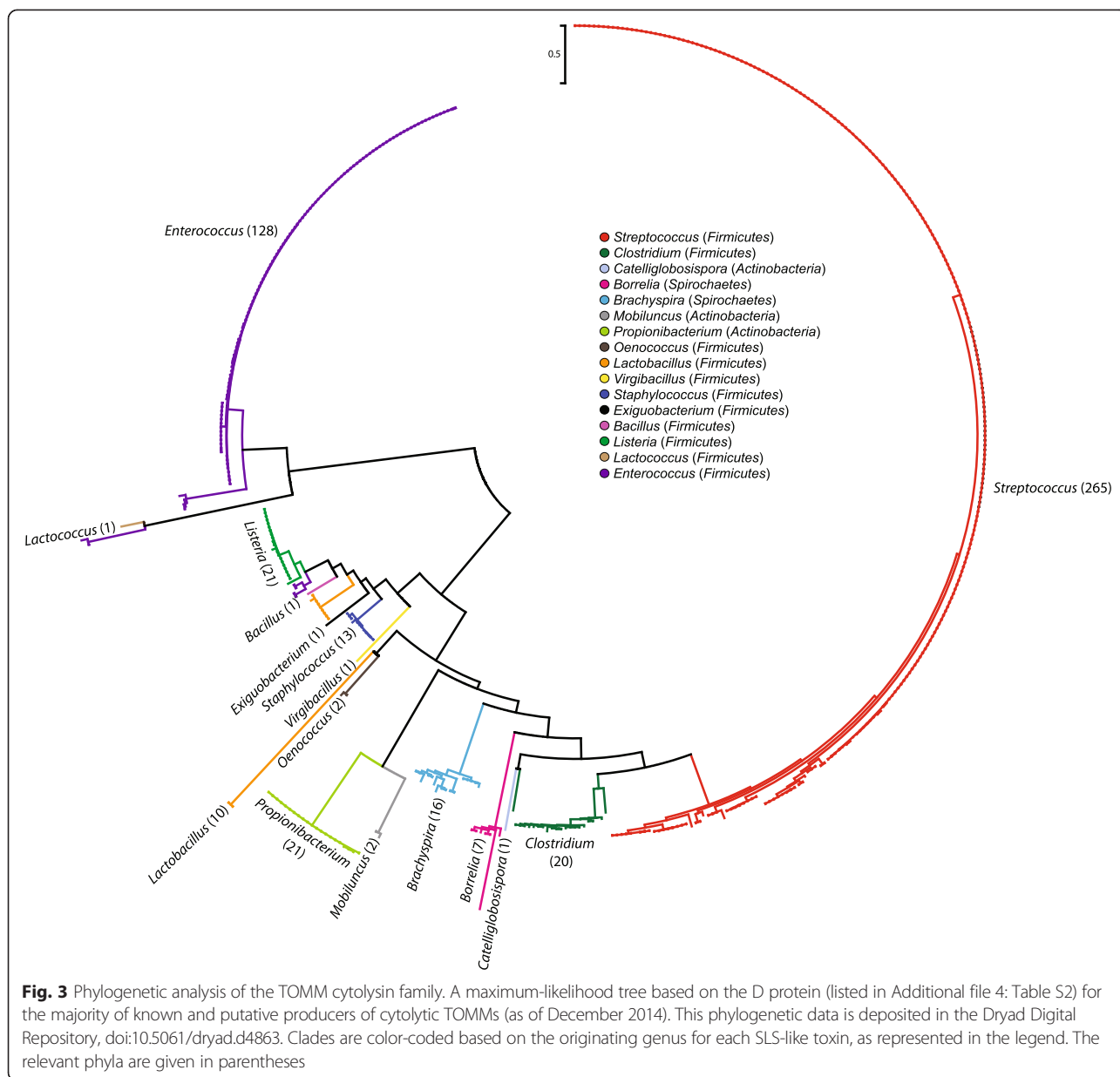
The retention of activity by such drastically C-terminally truncated SLS derivatives expressed in *S. pyogenes*  $\Delta$ sagA stands in contrast to alanine substitution studies that implicated several of the “missing” residues in SLS activity by both expression in *S. pyogenes*  $\Delta$ sagA and by modification using purified enzymes (Additional file 1: Figure S1B) [7, 22]. While a K53A mutation eliminated SLS hemolytic activity from *S. pyogenes* [22], our truncation series demonstrated that its removal is not detrimental to activity (Fig. 2b). Our results are consistent with the existence of natural SLS variants that lack a terminal lysine (Additional file 1: Figure S1A). Moreover, known SLS-like toxins encoded by non-*Streptococcus* species also lack terminal lysines (Fig. 1b). Even more striking is our observation that peptides that lack S39 retain cytolytic activity (Fig. 2b). S39 was identified as a critical residue because *S. pyogenes*  $\Delta$ sagA expressing SagA-S39A was devoid of cytolytic activity and avirulent in a mouse infection model [7] (Additional file 1: Figure S1B). As before, this position is not conserved in the SLS family (Additional file 1: Figure S1A) or in SLS-like toxins encoded by non-*Streptococcus* species (Fig. 1b). Finally, many of our mutants that lack the unconserved residues S46 and S48 are hemolytic to levels indistinguishable from WT. Previous mutagenesis suggested that these positions contribute to the hemolytic properties of SLS both in *S. pyogenes* and when modified with purified enzymes [7]. S46 and S48 have also been shown to be cyclized when SLS is modified with its cognate purified biosynthetic enzymes [17], although it is not known if this holds

true for native SLS. Thus, many positions that were previously implicated in SLS activity by point mutation are not conserved across the SLS family and were herein found to be dispensable. In the absence of a complete structure for SLS or detailed mechanism of action, it is unclear why mutation of a residue can be more detrimental than removal, but it is possible that the “alanine-substituted” toxins adopt an unproductive topology that is incapable of lysing cells.

### Bioinformatics-guided discovery of novel TOMM cytolysins

Given that the number of bioinformatically identifiable TOMM biosynthetic clusters more than quadrupled since 2011 [8, 25], we sought to determine if naturally truncated precursor peptides are present in any novel SLS-like clusters. Our genome mining effort revealed TOMM clusters encoding SagB, –C and –D homologs in the vicinity of SagA homologs in *Firmicutes* genera not previously known to possess such clusters (i.e. *Bacillus*, *Lactobacillus*, *Enterococcus*, *Exiguobacterium*, *Oenococcus* and *Virgibacillus*) (Additional file 2: Figure S2A and Additional file 3: Table S1). Interestingly, a number of *Actinobacteria* (*Mobiluncus* and *Propionibacterium*) and *Spirochaetes* (*Borrelia* and *Brachyspira*) encode SLS-like clusters (Additional file 2: Figure S2A and Additional file 3: Table S1). The *Brachyspira* TOMM clusters were independently noted in a recent publication [26]. We hypothesized that the newly-annotated SLS-like precursors (Additional file 2: Figure S2B) represent novel TOMM cytolysins since the sequence of a TOMM precursor dictates the function of the natural product after posttranslational modification [8, 6]. Moreover, while the novel SagA-like precursor peptides were of variable length, all contained the aforementioned NPH region. Our identification of these putative SLS-like cytolysins greatly expands the TOMM cytolysin family (Fig. 3 and Additional file 4: Table S2) and suggests that the TOMM cytolysins are not confined to *Firmicutes*.

Of particular interest were the three potential SLS-like TOMM biosynthetic clusters we discovered among the 23 published *Bbsl* genomes (Fig. 4a; hereafter referred to as “Bor TOMM clusters”), namely in *B. afzelii* PKO [GenBank: CP002947] [27], *B. valaisiana* VS116 [GenBank: CP001442] [28] and *B. spielmanii* A14S [GenBank: CP001465] [28]. The novel *Bbsl*-encoded precursor peptides were SagA homologs with core regions of similar length to our “minimal cytolytic unit” for SLS (Fig. 4b). We preliminarily classified the putative *Bbsl* SLS-like products as TOMM cytolysins based on sequence similarity to SLS, with support from a maximum likelihood tree of a subset of the TOMM family based on the associated D proteins (Fig. 4c). Thus, we assigned names to the homologous genes within the



clusters following the SLS nomenclature, with *borA* encoding the precursor peptide (refers collectively to *bafzA* from *B. afzelii* PKo, *bvalA* from *B. valaisiana* VS116, and *bspiA* from *B. spielmanii* A14S; Fig. 4b), *borB* encoding the dehydrogenase and *borC/D* encoding the cyclodehydratase and so forth.

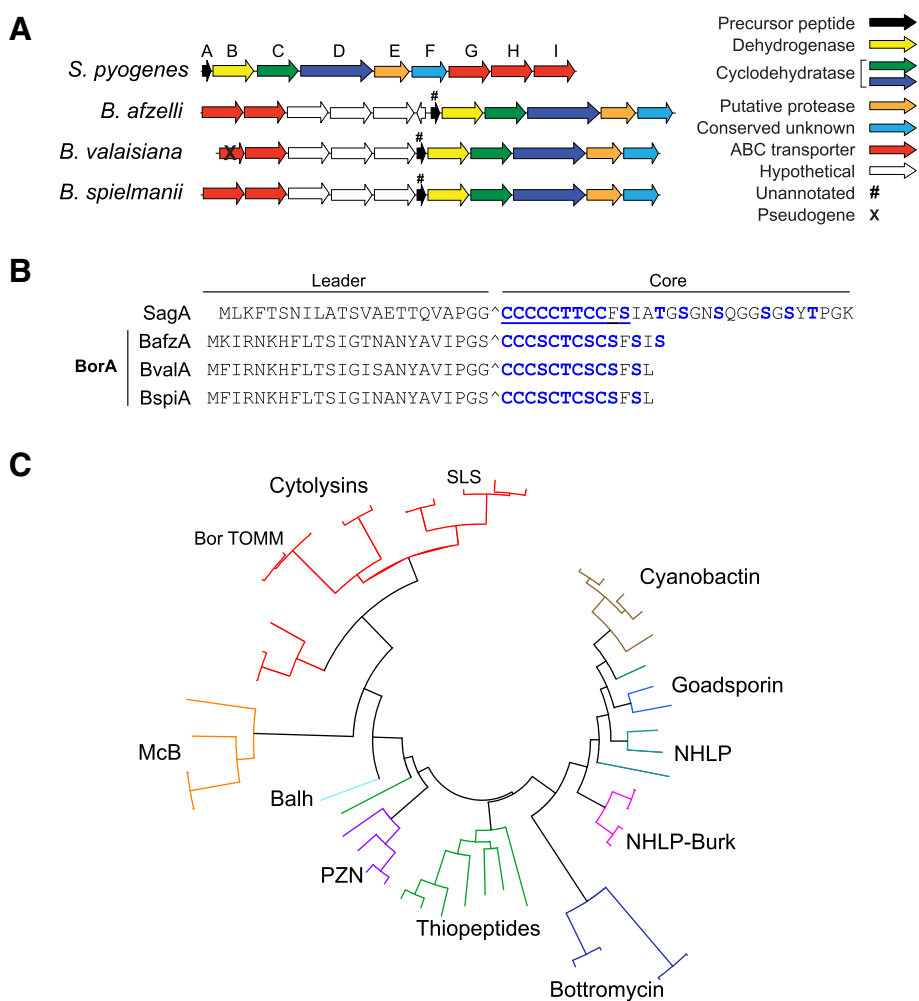
**SLS-like TOMM biosynthetic genes are widely distributed among *Bbsl***

We noted that the Bor TOMM clusters identified in *B. afzelii* PKo, *B. valaisiana* VS116 and *B. spielmanii* A14S were present on lp28-8 plasmids (Additional file 5: Figure S3). These plasmids exhibit 7-9 % nucleotide sequence divergence from each other, which is consistent

with that observed on the chromosome and other linear plasmids in these species [29]. Moreover, the G + C content of the cluster is 25-26 % in all three species, which is typical for *Bbsl* plasmids. It thus appears that the Bor TOMM cluster did not arrive into the genus recently by horizontal transfer, but has likely been present for a long time, diverging in parallel with the chromosome approximately when the three species diverged.

We hypothesized that the three Bor TOMM clusters identified in *Bbsl* genomes are an underestimation of the true prevalence given that *Bbsl* are prone to losing plasmids when cultured *in vitro* [30, 31]. In order to estimate the distribution of Bor TOMM clusters in *Bbsl*, we performed a PCR screen for the associated biosynthetic





**Fig. 4** Gene cluster organization and precursor peptide sequences of SLS and Bor TOMM, and phylogenetic analysis of the TOMM family. **a** Lettering corresponds to the SLS operon “sag” genes. The proposed function of each gene is color-coded according to the legend. **b** BorA refers collectively to BafzA from *B. afzelli* PKo, BvalA from *B. valaisiana* VS116, and BspiA from *B. spielmanii* A14S. Potentially modified residues are shown in blue. The predicted leader cleavage sites are indicated with a caret. The minimal core region required for hemolytic activity of SLS in *S. pyogenes* ΔsagA (SagA<sup>24–34</sup>) is underlined. **c** A maximum-likelihood tree of a representative sample of TOMMs based on the D protein from each cluster. This phylogenetic data is deposited in the Dryad Digital Repository, doi:10.5061/dryad.d4863. Clades are color-coded based on the predicted class of TOMM natural product. NHLP, nitrile hydratase leader peptide; NHLP-Burk, NHLP from *Burkholderia*; PZN, plantazolicin; Balh, uncharacterized TOMM from *Bacillus* sp. Al Hakam; McB, microcin B from *Gammaproteobacteria*; SLS, streptolysin S; Bor TOMM, putative SLS-like cytolysin from *Bbsl*

genes in 140 genomic DNA samples from tick or human patient isolates (Additional file 6: Table S3). Seven *Bbsl* species were represented, originating from North America and 13 countries in Europe and Asia. Because of the similarity among the VS116, PKo, and A14S Bor TOMM cluster sequences, primers specific to *bvalB*, -C and -D were used to detect *borB*, -C and -D in the isolates (Additional file 7: Figure S4 and Additional file 8: Table S4).

Our results reveal that Bor TOMM biosynthetic genes are present in select *B. afzelli*, *B. spielmanii*, *B. valaisiana*, *B. lusitanae*, and *B. garinii* isolates (Additional file 6: Table S3, summarized in Table 1). In line with the well-known plasmid instability of *Borrelia* plasmids, we observed cases of passage-dependent amplification of Bor

TOMM genes (Additional file 6: Table S3). While all three biosynthetic genes could be amplified from some of the aforementioned strains, there were many instances where only one or two biosynthetic genes were detected (Table 1 and Additional file 6: Table S3). Overall, *borC* was successfully amplified more frequently than *borB* or *borD*, likely due to higher conservation at the extreme 5’ and 3’ ends where the primers were designed to anneal (Additional file 7: Figure S4). Since mutational inactivation of nonessential genes is quite common on *Bbsl* linear plasmids, another possibility is that some target genes are too divergent to detect with the primers employed [32].

In contrast, Bor TOMM genes were absent from all *B. burgdorferi* sensu stricto (*Bbss*) and *B. bavariensis*

**Table 1** The Bor TOMM biosynthetic cluster is present among diverse *Bbsl* isolates

<i>Bbsl</i> species	No. of strains screened	PCR Positive			All
		<i>borB</i>	<i>borC</i>	<i>borD</i>	
<i>Bbss</i>	24	0	0	0	0
<i>B. garinii</i>	26	0	5	0	0
<i>B. afzelii</i>	47	26	34	19	14
<i>B. spielmanii</i>	7	5	5	5	5
<i>B. bavariensis</i>	13	0	0	0	0
<i>B. valaisiana</i>	21	3	15	3	3
<i>B. lusitaniae</i>	2	0	2	2	0

Positive PCR hits for Bor TOMM biosynthetic genes in *Bbsl* isolates. Strains originated in Europe and Asia, except 11 *Borrelia burgdorferi* sensu stricto (*Bbss*) from North America. "All" indicates the number of isolates testing positive for genes encoding all three putative heterocycle forming enzymes, BorBCD. More details are provided in Additional file 6: Table S3

isolates screened (Table 1 and Additional file 6: Table S3), suggesting that they do not possess a Bor TOMM cluster, or again, that the target genes are too divergent to detect. The fact that the Bor TOMM is not present in the 13 *Bbss* published genomes [33] suggests that the former is likely true for this species. Notably, *Bbss* has been reported to produce a number of hemolytic factors [34, 35].

Mapping the distribution of a subset of the Bor TOMM cluster data onto a maximum likelihood tree, which was generated from a multilocus sequence typing (MLST) analysis, further supports our hypothesis that the Bor TOMM cluster has long been present in *Borrelia* (Additional file 9: Figure S5). The combined effects of a non-exhaustive PCR screen with non-degenerate primers and the unavoidable plasmid loss upon cultivation means that data reported here represent the minimum distribution and prevalence of the Bor TOMM cluster in *Bbsl*. However, these data demonstrate that the newly-identified Bor TOMM cluster is underrepresented in available genome sequences.

#### The SLS-like peptides from *Borrelia* are potential cytolysins

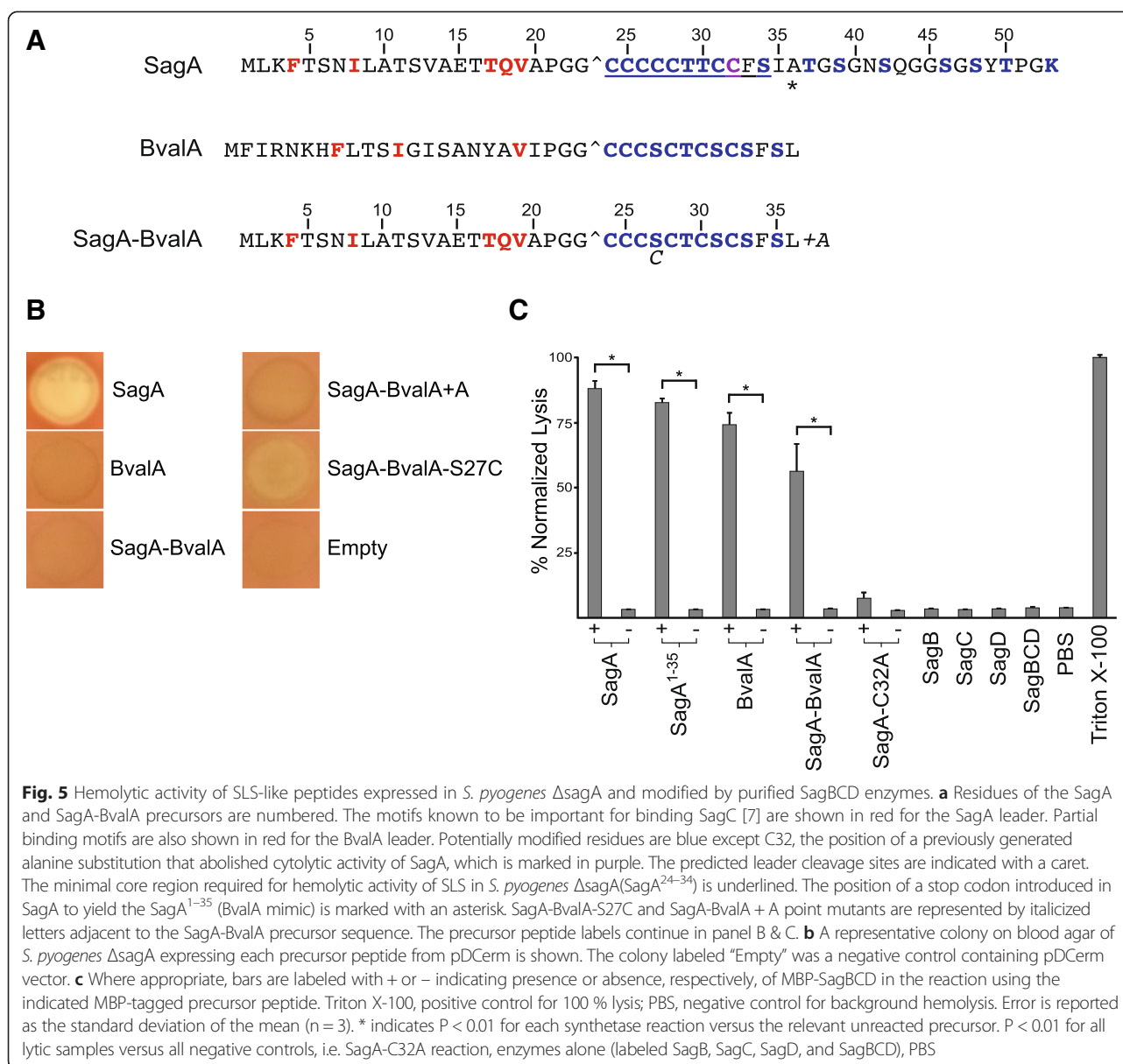
Given that cytolytic TOMM production has been implicated in the virulence of some pathogens [7, 22, 18], coupled with the prevalence of SLS-like TOMM clusters in *Bbsl*, we decided to study the cytolytic potential of one of the naturally C-terminally truncated SLS-like peptides from *Bbsl*. In light of the complexity of *Borrelia* cultivation requirements, the aforementioned tendency to lose plasmids when cultured and inefficient genetic tools [36, 37], we opted to take a reductionist approach and assess the cytolytic potential of BvalA by heterologous expression in *S. pyogenes*  $\Delta$ sagA. Importantly, there is precedent for this approach, e.g. the SLS-like precursor from *C. botulinum* (Fig. 1b) successfully complemented *S. pyogenes*  $\Delta$ sagA [7] and was later confirmed to function as a cytolysin in the context of a

native producer [17]. BvalA bears ~70 % amino acid similarity to SagA (Fig. 5a) and the SagBCD proteins are ~50 % similar to their BvalBCD equivalents. However, no hemolytic activity was observed upon heterologous expression of the non-cognate substrate BvalA in *S. pyogenes*  $\Delta$ sagA (Fig. 5b).

Substrate binding during TOMM biosynthesis occurs via recognition of key motifs within N-terminal leader region of the precursor peptide by the C-protein component of the synthetase [25, 7]. Although partial binding motifs appear to be present in the BorA leader peptides (Fig. 5a), we cannot rule out the possibility that BvalA was not bound by the *S. pyogenes* biosynthetic machinery during heterologous expression. In light of this, we generated a chimeric construct encoding the cognate SagA leader peptide fused to the non-cognate BvalA core peptide (SagA-BvalA; Fig. 5a). Such a strategy has been exploited to replace highly divergent leader peptides with the SagA leader to allow native interaction with the heterocycle-forming synthetase [7]. In this way, hemolytic activity was demonstrated for the SLS-like core from *S. aureus* (Fig. 1b) upon expression in *S. pyogenes*  $\Delta$ sagA [7]. However, SagA-BvalA was not hemolytic under identical conditions (Fig. 5b). Co-expression of rare streptococcal tRNAs [38] also failed to yield hemolysis from BvalA or SagA-BvalA, suggesting that their inactivity was not due to codon-usage bias during heterologous expression.

Returning to the native SagA precursor peptide, we noted a dramatic decrease in activity for SagA<sup>1-35</sup> (which could be considered a BvalA mimic; Fig. 5a), compared to SagA<sup>1-36</sup> (Fig. 2b). In case the hemolytic activity of SagA-BvalA was just below the limit of detection of this assay, we appended an alanine to the C-terminus of SagA-BvalA (SagA-BvalA + A is akin to SagA<sup>1-36</sup>); however, hemolysis was again not observed (Fig. 5b). While the SagA core begins with a stretch of five contiguous cysteine residues, there is a serine residue at the fourth position of the comparable region of BvalA (Fig. 5a). We thus created SagA-BvalA-S27C, which partly rescued activity (Fig. 5b), causing hemolysis only directly beneath the colony compared to the zone of clearing observed in strains with high hemolytic activity. Synthetase reactions with reconstituted TOMM biosynthetic enzymes have established that cysteine is cyclized more rapidly than serine and threonine and several TOMM synthetases are known to be selective for cysteine [39, 40]. We speculate that replacing serine with cysteine partly ameliorates a bottleneck for SagBCD in processing an unnatural substrate.

The lack of activity from BvalA-derived substrates upon expression in *S. pyogenes*  $\Delta$ sagA prompted us to assess cytolytic potential using purified SagBCD enzymes. Previously, SLS-like cytolysins with no detectable activity when



expressed in *S. pyogenes*  $\Delta$ sagA were converted to cytolysins by this approach [7]. Numerous factors contribute to this, but perhaps most importantly, high concentrations of reactants can be employed to increase the heterocyclization yield on non-optimal substrates. Additionally, this cell-free system obviates the need for export/proteolysis machinery to generate lytic entities [6, 7]. Attempts to obtain sufficiently pure, full-length and active recombinant BvalBCD were unsuccessful; however, after treatment with the SagBCD homologs (Additional file 3: Table S1C), BvalA and SagA-BvalA were both converted into cytolysins with activity comparable to SagA and SagA<sup>1-35</sup> (Fig. 5c). Omission of either the peptide substrate or SagBCD resulted in no lysis, similar to the non-lytic C32A mutant of SagA [7]. These data indicated that the BvalA

precursor peptide has the potential to be converted into a cytolysin under certain conditions.

The majority of non-cognate and mutant precursor peptides that become cytolytic upon treatment with purified SagBCD were also lytic when constitutively expressed in *S. pyogenes*  $\Delta$ sagA [7]. However, there is precedent for the failure of *bona fide* SLS-like precursors to restore hemolysis in *S. pyogenes*  $\Delta$ sagA, e.g. as was the case with listeriolysin S (Fig. 1b) [18, 7]. The contrasting results for BvalA and SagA-BvalA (Fig. 5b and c) are reminiscent of the chimeric *L. monocytogenes* substrate, SagA-LlsA, which was inactive in *S. pyogenes*  $\Delta$ sagA but was transformed into a cytolysin by purified SagBCD [7]. These results underscore the strict requirements for efficient enzymatic processing and toxin export in *S.*



*pyogenes*. Nevertheless, the ability of purified SagBCD to convert BvalA into a cytolysin, coupled with the retention of activity by truncated SagA peptides upon functional expression, suggests that the short SagA homologs found in *Bbsl* may function as cytolysins in the context of their native producers. However, whether this activity is the primary function of the Bor TOMM (if produced) requires additional study.

## Conclusions

The results of our SagA truncation series indicate that, of the 30 predicted to comprise the core peptide, the first 11 residues (SagA<sup>24–34</sup>) are sufficient for detectable hemolytic activity of SLS when expressed in *S. pyogenes*  $\Delta$ sagA. Thus, our data indicates that the C-terminus of SagA is largely dispensable and points to the NPH region as central to SLS functionality, consistent with the conservation of this motif in natural SLS variants as well as the greater TOMM cytolysin family. Similar regions consisting of contiguous heterocyclizable residues exist in the newly-described SLS-like precursors encoded in multiple genera within *Firmicutes*, *Spirochaetes* and *Actinobacteria*. Although the ability of BvalA and SagA<sup>1–35</sup> to be matured into cytolysins by purified SagBCD suggests that the “minimal cytolytic unit” may be a general feature of TOMM cytolysins, additional study will be required to determine if this holds true for all family members, especially those with longer stretches of heterocyclizable residues (e.g. listeriolysin S). In order to comprehend our observation that much of the hemolytic activity of SLS can be attributed to a mere 11 residues, a complete chemical structure of SLS and greater molecular insight into the interaction of SLS with the cellular membrane will be required.

Our identification of novel SLS-like biosynthetic clusters greatly expands the TOMM cytolysin family. Of particular interest is our discovery that many pathogenic *Bbsl* harbor the genetic capacity to produce naturally truncated SLS-like toxins. Although confirmation is needed, the most probable function for the SLS-like biosynthetic clusters in *Bbsl* is to generate a cytolytic factor, akin to the virulence-enhancing SLS of *S. pyogenes*. Thus, the hypothetical “borreliolysin S” toxin we identify herein warrants further investigation as it would represent, to the best of our knowledge, the first post-translationally modified natural product from any Spirochaete.

In summary, this work provides evidence that the core NPH motif represents the defining feature of the SLS-like TOMM family, as well as providing a foundation for studies of the potential utilization of cytolytic TOMMs by the diverse organisms described herein.

## Methods

### Bacterial culture conditions

Strains and plasmids used in this study are listed in Additional file 10: Table S5, except *Bbsl* strains which

are listed in Additional file 6: Table S3. *S. pyogenes* were grown at 37 °C in Todd–Hewitt broth or agar plates, or on plates of 5 % sheep blood in tryptic soy agar (Hardy Diagnostics) when determining hemolytic activity by functional expression. For antibiotic selection, 5  $\mu$ g ml<sup>-1</sup> erythromycin was used. *E. coli* strains were grown at 37 °C with aeration in Luria–Bertani broth or on Luria–Bertani agar plates; antibiotic selection utilized 500  $\mu$ g ml<sup>-1</sup> erythromycin and 50  $\mu$ g ml<sup>-1</sup> kanamycin where relevant.

### Genome mining for novel SLS-like clusters

The majority of TOMM clusters encode a precursor peptide adjacent to SagB, –C and –D homologs [8, 10], enabling their bioinformatic detection. Thus, novel SLS-like clusters were identified by BLAST-P of known SLS-like D proteins (SagD and homologs from *C. botulinum*, *S. aureus*, *L. monocytogenes*) as the query sequence. After identification of BvalD, BmurD, LactD, and EnduD, these sequences were used as a query sequence to find other homologs, with a BLAST e-value of 1e-30 as a cutoff. Subsequently, homologs of other *sag* genes were identified in the local genomic region of a single representative cluster from each genus. Manual searches were performed in the intergenic regions of the representative clusters for short, unannotated genes encoding recognizable TOMM precursors, which are usually overlooked by gene-identification algorithms. Because of inherent duplications in RefSeq, only a single representative sequence was used from organisms with no strain information. Genome clusters with low e-values were annotated manually for precursor peptides and genomic regions that resembled known cytolysins.

### Maximum likelihood phylogenetic analysis

The D proteins of a diverse set of putative TOMM cytolysin producers were analyzed because it is known that the B and C proteins can sometimes be omitted from TOMM biosynthetic gene clusters, whereas D proteins are always present [10]. Further, the B and C proteins are homologous to non-TOMM dehydrogenases and the E1-ubiquitin activating family of enzymes, and thus are prone to bioinformatic false-positives. Thus, a D protein alignment was created using the standard parameters of ClustalW [41] and then a maximum likelihood tree was made using the standard parameters of Molecular Evolutionary Genetics Analysis (MEGA) [42]. MLST phylogenetic analysis was generated in MEGA 5.1 using concatenated sequences of eight chromosomally-located housekeeping genes (*clpA*, *clpX*, *nifS*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) [43] using the maximum likelihood method with 1,000 bootstrap repeats, the GTR model, uniform rates among sites and nearest-neighbor-interchange.

### PCR screen for Bor TOMM biosynthetic genes

Genomic DNA of cultured *Bbsl* isolates was purified using either a Qiagen DNeasy blood and tissue kit or a High Pure PCR Template Preparation Kit (Roche Diagnostics) [44, 45]. Total DNA from field-collected questing ticks was extracted using alkaline hydrolysis [46]. Genomic DNA from each *Bbsl* isolate was tested for the presence of *borB*, *-C*, and *-D* by *Taq* PCR with primers specific to VS116 (Additional file 8: Table S4). Amplification of a product of appropriate length as determined by agarose gel electrophoresis was considered a positive hit. Sanger sequencing confirmed the identity of PCR amplicons from each species. Primers that effectively amplified *Bbsl* 16S rRNA gene sequences were used to verify that sufficient DNA was present in all samples.

### Preparation of plasmids for *S. pyogenes* transformation

The chemically synthesized genes (Integrated DNA Technologies) encoding BvalA and SagA-BvalA were subcloned into pDCerm as previously described [7]. Stop codons were introduced by site-directed mutagenesis. Mutant candidates were miniprepmed and verified by DNA sequencing using pDCerm\_SeqMCS\_F. Primers are listed in Additional file 8: Table S4. In order to obtain sufficient plasmid DNA for transformation of *S. pyogenes*  $\Delta$ sagA, each pDCerm construct was maxiprepmed using an E.Z.N.A. Plasmid Maxi Kit (Omega Bio-tek).

### *S. pyogenes* transformation

*S. pyogenes*  $\Delta$ sagA [22] was rendered electrocompetent using a modified version of a previously published method [47]. Briefly, *S. pyogenes*  $\Delta$ sagA was grown to an OD<sub>600</sub> of 0.35 in 50 ml Todd–Hewitt broth with 0.6 % (w/v) glycine. Cells were washed three times with cold 0.625 M sucrose (pH 4) and resuspended in 300  $\mu$ l of the same buffer with 20 % (v/v) glycerol. 50  $\mu$ l of cells was equilibrated with ~1  $\mu$ g of plasmid DNA on ice for 30 min and electroporated at 1.65 kV with a BioRad Micropulser. Immediately, cells were added to 250  $\mu$ l Todd–Hewitt broth with 0.25 M sucrose and recovered for 2 h at 37 °C, before plating. The *S. pyogenes*-nature of transformants [48] and presence of pDCerm were verified by PCR (Additional file 8: Table S4).

### Reaction of SLS-like peptides with purified SagBCD

All proteins were expressed with maltose-binding protein (MBP) fused to the N-terminus. SagA, SagA-C32A [7], SagB, SagC and SagD were prepared as previously described [6]. The genes encoding BvalA and SagA-BvalA were subcloned into pET28b-MBP as previously described [6]. Site-directed mutagenesis was performed to yield pET28b-MBP-SagA<sup>1–35</sup> (Additional file 8: Table S4). Precursor peptides were expressed from *E. coli* BL21(DE3)-RIPL (Stratagene) for 1 h at 30 °C after

induction with 1 mM IPTG at OD<sub>600</sub> of 0.6. Following standard lysis procedures, each protein was purified by amylose affinity chromatography [6]. MBP-SagB, *-C* and *-D* (10  $\mu$ M each) were combined with the relevant MBP-tagged precursor peptide (40  $\mu$ M) and allowed to react in synthetase buffer as previously described [6]. The products of the synthetase reactions were applied to washed erythrocytes and lysis assessed as previously described [6]. Hemoglobin absorbance was measured at 410 nm on a Tecan Infinite<sup>®</sup> 200 PRO plate reader. Values were normalized to a Triton X-100 positive control. Phosphate buffered saline (PBS) served as a negative control.

### Availability of supporting data

The data sets supporting the results of this article are available in the Dryad Digital Repository (<http://datadryad.org/>), doi:10.5061/dryad.d4863.

### Additional files

**Additional file 1: Figure S1.** Alignment of experimentally validated streptococcal SLS variants and SLS structure-activity relationships. (A) Alignment using Clustal Omega [49] reveals that the SLS core region possesses a highly conserved N-terminus containing 9–10 contiguous heterocyclizable residues (underlined), while the C-terminus is variable in terms of both identity of residues and length. This phylogenetic data is deposited in the Dryad Digital Repository, doi:10.5061/dryad.d4863. The putative leader peptide cleavage sites are shown as carets. Residues of SagA from *S. pyogenes* are numbered. C-terminal residues where alanine substitutions were previously installed and that are specifically referred to in the article are marked in red (see panel B). (B) Summary of cytolytic activity of SagA core peptide mutants measured by expression in *S. pyogenes*  $\Delta$ sagA. Activity on blood agar equal to complementation with wild-type SagA is designated as +++, 30–70 % activity is ++, less than 30 % activity is +, and non-detectable activity is –. C-terminal mutations specifically referred to in the article are marked in red. Mutants were expressed from pDCerm under control of a constitutive promoter [7] or from pAD under control of the native *sagA* promoter [22].

**Additional file 2: Figure S2.** Gene cluster organization and precursor peptide sequences of predicted SLS-like TOMM biosynthetic gene clusters. (A) One example is given for each genus with strain abbreviations as per Additional file 3: Table S1A. Lettering corresponds to the SLS operon “sag” genes. The proposed function of each gene is color-coded according to the legend. In the case of *Lactococcus* and *Catelliglobospora* (included in Fig. 3), the protein sequences are not associated with a genome in GenBank, making it difficult to confirm all surrounding genes; thus, a representative cluster organization was not given. Those clusters identified in *Borrelia* are depicted in Fig. 4 and Additional file 5: Figure S3. (B) Potentially modified residues are shown in blue. The predicted leader cleavage sites are indicated with a caret.

**Additional file 3: Table S1.** Accession numbers and expectation values of BCD proteins from representative SLS-like TOMM clusters. (A) Strains used for the similarity analysis and the corresponding abbreviations (continued in panels B and C). (B) GenBank accession numbers (ncbi.nlm.nih.gov) of the B, C and D proteins used for the analysis. (C) BLAST e-values for the designated proteins upon comparison with the *B. valaisiana* VS116 homolog. E-values were determined using BLAST-P with standard settings and limiting the searched organisms to include only the strains in panel A, except *B. valaisiana* VS116.

**Additional file 4: Table S2.** List of accession identifiers for D proteins of putative TOMM cytolytic clusters. The genus, species, strain, and GenBank accession identifiers are given for all D proteins used in the phylogenetic analysis (Fig. 3), with inherent duplications from GenBank RefSeq annotations deleted.

**Additional file 5: Figure S3.** Open reading frame (ORF) diagrams for *Borrelia* lp28-8 plasmids. Four published *Bbsl* genomes harbor plasmids in the lp28-8 compatibility group [50]. As is typical for *Bbsl* linear plasmids [51], the lp28-8 plasmids are mosaically related; gray areas between maps denote regions of sequence similarity. lp28-8 from *B. burgdorferi* 94a lacks the Bor TOMM biosynthetic cluster so is not further discussed. The ORFs of the Bor TOMM clusters of *B. spielmanii* A14S, *B. valaisiana* VS116 and *B. afzelii* PKo are color-coded as in Fig. 1A; white arrows are hypothetical or have functions unrelated to the Bor TOMM biosynthetic cluster. The numbers above an ORF give the Pfam designation. Names of the ORFs known from type strain *B. burgdorferi* B31 are labeled “b31\_” and are shown below the maps. Each lp28-8 carries a truncated *vls* cassette region, which allows alteration of the antigenic properties of VlsE outer surface proteins, a key pathogenic strategy. An “X” over an ORF indicates a pseudogene. In VS116, the ABC transporter gene has a substantial deletion and may be nonfunctional, suggesting that a different transporter performs export or that VS116 does not produce the Bor TOMM.

**Additional file 6: Table S3.** PCR screen for Bor TOMM biosynthetic genes. These results provide more detail on those given in Table 1. Strains marked in gray have published genomes. P# indicates number of passages where known. Male (M), female (F) or nymph (N) is indicated for tick isolates where known. In the case of human isolates, CSF indicates isolation from cerebrospinal fluid. Disease presentation is given where known: EM, erythema migrans; LD, Lyme disease; NB, neuroborreliosis; ACA, acrodermatitis chronica atrophicans. Source abbreviations: IP, Institute Pasteur; VF, Volker Fingerle; IS, Ira Schwartz; SC, Sherwood Casjens; RM, Richard Marconi; AS, Arno Schönberg; GM: Gabriele Margos; SB, Sven Bergstrom; PR, Patricia Rosa; UM, Ulrike Munderloh; FS, Franc Strle; AvD, Alje van Dam. <sup>1</sup>LGL Bayern, Germany. \**Bbsl* species group currently contains 21 confirmed and proposed species, including human pathogens, suspected pathogens and non-pathogenic species [52–54]. <sup>2</sup>The reported *B. afzelii* ACA-1 genome sequence does not contain Bor TOMM genes [27]; however, we detected *borBCD* in two of three ACA-1 strains of differing passage numbers. Presumably lp28-8 was present in the original ACA-1 isolate but was lost prior to the sequencing of its genome. This hypothesis is supported by a report of the sequence of ACA-1 *vls* cassette region, also missing from the published ACA-1 genome, indicating the presence of the lp28-8 in their culture [55]. <sup>3</sup>*borBCD* were not detected in passage 7 of a PKo strain that at passage 6 had its genome sequenced [27]. In contrast, *borBCD* were detected in another isolate of PKo at passage 10, again highlighting the well-known heterogeneous nature of *Bbsl* upon cultivation.

**Additional file 7: Figure S4.** Nucleotide alignments of Bor TOMM biosynthetic genes. *borB* (A), *borC* (B) and *borD* (C) from *Borrelia afzelii* PKo (Bafz), *B. spielmanii* A14S (Bspi), and *B. valaisiana* VS116 (Bval) were aligned using ClustalW. This phylogenetic data is deposited in the Dryad Digital Repository, doi:10.5061/dryad.d4863. Sites where PCR screening primers annealed are underlined. The primers used to detect *borB/C/D* (Table 1 and Additional file 6: Table S3) were based on *bvalB/C/D* from *B. valaisiana* VS116 (Additional file 8: Table S4). The number of mismatches per primer when compared to the two other sequenced examples of the gene is indicated in parentheses for each.

**Additional file 8: Table S4.** Primers used in this study. In all cases, *bvalA/B/C/D* or *BvalA/B/C/D* refers to the relevant TOMM genes or proteins from *B. valaisiana* VS116. Restriction endonuclease recognition sites are underlined. Lower-case letters indicate bases targeted for mutagenesis.

**Additional file 9: Figure S5.** Maximum likelihood tree of *Bbsl* strains used in the Bor TOMM PCR screen. The tree shows the relationships of a number of *Borrelia* strains, including a subset of the *Bbsl* strains included in our PCR screen. This phylogenetic data is deposited in the Dryad Digital Repository, doi:10.5061/dryad.d4863. A plus sign (+) next to the strain designation indicates that gene(s) from the Bor TOMM biosynthetic cluster (*borB/borC/borD*) was/were detected (Additional file 6: Table S3). WGS: whole genome sequence.

**Additional file 10: Table S5.** Strains and plasmids used in this study. In all cases, *bvalA/BvalA* refers to the TOMM gene/peptide from *B. valaisiana* VS116. The sequences of the relevant precursor peptides are shown in Figs. 2A and 5A.

## Abbreviations

SLS: Streptolysin S; TOMM: Thiazole/oxazole-modified microcin; NPH: N-terminal poly-heterocyclizable (region); *Bbsl*: *Borrelia burgdorferi* sensu lato; *Bbss*: *Borrelia burgdorferi* sensu stricto; MLST: Multilocus sequence typing; MEGA: Molecular Evolutionary Genetics Analysis; MBP: Maltose-binding protein; PBS: Phosphate buffered saline; ORF: Open reading frame.

## Competing interests

The author(s) declare that they have no competing interests.

## Authors' contributions

EMM designed and performed experiments and wrote the manuscript. SRC and GM provided critical reagents, performed bioinformatic analysis and contributed to the manuscript. CLC performed bioinformatic analysis and contributed to the manuscript. NAE performed experiments. VF & TM provided key reagents and contributed to the manuscript. DAM conceived the work, designed experiments and wrote the manuscript. All authors read and approved the final manuscript.

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## References

- Cunningham MW. Pathogenesis of group A streptococcal infections. Clin Microbiol Rev. 2000;13(3):470–511.
- Marmorek A. Le streptocoque et le sérum antistreptococcique. Ann Inst Pasteur. 1895;9:593–620.
- Todd EW. The differentiation of two distinct serologic varieties of streptolysin, streptolysin O and streptolysin S. J Pathol Bacteriol. 1938;47:423–45.
- Molloy EM, Cotter PD, Hill C, Mitchell DA, Ross RP. Streptolysin S-like virulence factors: the continuing *sagA*. Nat Rev Microbiol. 2011;9(9):670–81.
- Nizet V, Beall B, Bast DJ, Datta V, Kilburn L, Low DE, et al. Genetic locus for streptolysin S production by group A streptococcus. Infect Immun. 2000;68(7):4245–54.
- Lee SW, Mitchell DA, Markley AL, Hensler ME, Gonzalez D, Wohlrab A, et al. Discovery of a widely distributed toxin biosynthetic gene cluster. Proc Natl Acad Sci U S A. 2008;105(15):5879–84.
- Mitchell DA, Lee SW, Pence MA, Markley AL, Limm JD, Nizet V, et al. Structural and functional dissection of the heterocyclic peptide cytotoxin streptolysin S. J Biol Chem. 2009;284(19):13004–12.
- Melby JO, Nard NJ, Mitchell DA. Thiazole/oxazole-modified microcins: complex natural products from ribosomal templates. Curr Opin Chem Biol. 2011;15(3):369–78.
- Oman TJ, van der Donk WA. Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nat Chem Biol. 2010;6(1):9–18.
- Arnison PG, Bibb MJ, Bierbaum G, Bowers AA, Bugni TS, Bulaj G, et al. Ribosomally synthesized and post-translationally modified peptide natural



- products: overview and recommendations for a universal nomenclature. *Nat Prod Rep*. 2013;30(1):108–60.
11. Pei J, Mitchell DA, Dixon JE, Grishin NV. Expansion of type II CAAX proteases reveals evolutionary origin of gamma-secretase subunit APH-1. *J Mol Biol*. 2011;410(1):18–26.
  12. Maxson T, Deane CD, Molloy EM, Cox CL, Markley AL, Lee SW, et al. HIV protease inhibitors block streptolysin S production. *ACS Chem Biol*. 2015;10(5):1217–26.
  13. Humar D, Datta V, Bast DJ, Beall B, De Azavedo JC, Nizet V. Streptolysin S and necrotising infections produced by group G streptococcus. *Lancet*. 2002;359(9301):124–9.
  14. Fuller JD, Camus AC, Duncan CL, Nizet V, Bast DJ, Thune RL, et al. Identification of a streptolysin S-associated gene cluster and its role in the pathogenesis of *Streptococcus iniae* disease. *Infect Immun*. 2002;70(10):5730–9.
  15. Flanagan J, Collin N, Timoney J, Mitchell T, Mumford JA, Chanter N. Characterization of the haemolytic activity of *Streptococcus equi*. *Microb Pathogenesis*. 1998;24(4):211–21.
  16. Tabata A, Nakano K, Ohkura K, Tomoyasu T, Kikuchi K, Whiley RA, et al. Novel twin streptolysin S-like peptides encoded in the *sag* operon homologue of beta-hemolytic *Streptococcus anginosus*. *J Bacteriol*. 2013;195(5):1090–9.
  17. Gonzalez DJ, Lee SW, Hensler ME, Markley AL, Dahesh S, Mitchell DA, et al. Clostridiolysin S, a post-translationally modified biotoxin from *Clostridium botulinum*. *J Biol Chem*. 2010;285(36):28220–8.
  18. Cotter PD, Draper LA, Lawton EM, Daly KM, Groeger DS, Casey PG, et al. Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes*. *PLoS Pathog*. 2008;4(9):e1000144.
  19. Clayton EM, Daly KM, Guinane CM, Hill C, Cotter PD, Ross PR. Atypical *Listeria innocua* strains possess an intact LIPI-3. *BMC Microbiol*. 2014;14(1):58.
  20. Clayton EM, Hill C, Cotter PD, Ross RP. Real-time PCR assay to differentiate Listeriolysin S-positive and -negative strains of *Listeria monocytogenes*. *Appl Environ Microbiol*. 2011;77(1):163–71.
  21. Carr A, Sledjeski DD, Podbielski A, Boyle MD, Kreikemeyer B. Similarities between complement-mediated and streptolysin S-mediated hemolysis. *J Biol Chem*. 2001;276(45):41790–6.
  22. Datta V, Myskowski SM, Kwinn LA, Chiem DN, Varki N, Kansal RG, et al. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. *Mol Microbiol*. 2005;56(3):681–95.
  23. Radolf JD, Caimano MJ, Stevenson B, Hu LT. Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nat Rev Microbiol*. 2012;10(2):87–99.
  24. Stanek G, Wormser GP, Gray J, Strle F. Lyme borreliosis. *Lancet*. 2012;379(9814):461–73.
  25. Dunbar KL, Chekan JR, Cox CL, Burkhart BJ, Nair SK, Mitchell DA. Discovery of a new ATP-binding motif involved in peptidic azoline biosynthesis. *Nat Chem Biol*. 2014;10(10):823–9.
  26. Letzel AC, Pidot SJ, Hertweck C. Genome mining for ribosomally synthesized and post-translationally modified peptides (RiPPs) in anaerobic bacteria. *BMC Genomics*. 2014;15(1):983.
  27. Casjens SR, Mongodin EF, Qiu WG, Dunn JJ, Luft BJ, Fraser-Liggett CM, et al. Whole-genome sequences of two *Borrelia afzelii* and two *Borrelia garinii* Lyme disease agent isolates. *J Bacteriol*. 2011;193(24):6995–6.
  28. Schutzer SE, Fraser-Liggett CM, Qiu WG, Kraiczky P, Mongodin EF, Dunn JJ, et al. Whole-genome sequences of *Borrelia bisettii*, *Borrelia valaisiana*, and *Borrelia spielmanii*. *J Bacteriol*. 2012;194(2):545–6.
  29. Mongodin EF, Casjens SR, Bruno JF, Xu Y, Drabek EF, Riley DR, et al. Inter- and intra-specific pan-genomes of *Borrelia burgdorferi* sensu lato: genome stability and adaptive radiation. *BMC Genomics*. 2013;14:693.
  30. Biskup UG, Strle F, Ruzic-Sabljić E. Loss of plasmids of *Borrelia burgdorferi* sensu lato during prolonged *in vitro* cultivation. *Plasmid*. 2011;66(1):1–6.
  31. Stewart PE, Byram R, Grimm D, Tilly K, Rosa PA. The plasmids of *Borrelia burgdorferi*: essential genetic elements of a pathogen. *Plasmid*. 2005;53(1):1–13.
  32. Casjens S. *Borrelia* genomes. In: Saier M, Garcia-Lara G, editors. The spirochetes: molecular and cellular biology. Norfolk UK: Horizon Scientific Press; 2001. p. 75–85.
  33. Schutzer SE, Fraser-Liggett CM, Casjens SR, Qiu WG, Dunn JJ, Mongodin EF, et al. Whole-genome sequences of thirteen isolates of *Borrelia burgdorferi*. *J Bacteriol*. 2011;193(4):1018–20.
  34. Williams LR, Austin FE. Hemolytic activity of *Borrelia burgdorferi*. *Infect Immun*. 1992;60(8):3224–30.
  35. Shaw DK, Hyde JA, Skare JT. The BB0646 protein demonstrates lipase and haemolytic activity associated with *Borrelia burgdorferi*, the aetiological agent of Lyme disease. *Mol Microbiol*. 2012;83(2):319–34.
  36. Hyde JA, Weening EH, Skare JT. Genetic transformation of *Borrelia burgdorferi*. *Curr Protoc Microbiol*. 2011;Chapter 12:Unit 12C 4.
  37. Chaconas G, Norris SJ. Peaceful coexistence amongst *Borrelia* plasmids: getting by with a little help from their friends? *Plasmid*. 2013;70(2):161–7.
  38. Lee SF, Li YJ, Halperin SA. Overcoming codon-usage bias in heterologous protein expression in *Streptococcus gordonii*. *Microbiology*. 2009;155(Pt 11):3581–8.
  39. Melby JO, Dunbar KL, Trinh NQ, Mitchell DA. Selectivity, directionality, and promiscuity in peptide processing from a *Bacillus* sp. AI Hakam cyclodehydratase. *J Am Chem Soc*. 2012;134(11):5309–16.
  40. Belshaw PJ, Roy RS, Kelleher NL, Walsh CT. Kinetics and regioselectivity of peptide-to-heterocycle conversions by microcin B17 synthetase. *Chem Biol*. 1998;5(7):373–84.
  41. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007;23(21):2947–8.
  42. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28(10):2731–9.
  43. Margos G, Vollmer SA, Cornet M, Garnier M, Fingerle V, Wilske B, et al. A new *Borrelia* species defined by multilocus sequence analysis of housekeeping genes. *Appl Environ Microbiol*. 2009;75(16):5410–6.
  44. Margos G, Gatewood AG, Aanensen DM, Hanincova K, Terekhova D, Vollmer SA, et al. MLST of housekeeping genes captures geographic population structure and suggests a European origin of *Borrelia burgdorferi*. *Proc Natl Acad Sci U S A*. 2008;105(25):8730–5.
  45. Schulte-Spechtel U, Fingerle V, Goettner G, Rogge S, Wilske B. Molecular analysis of decorin-binding protein A (DbpA) reveals five major groups among European *Borrelia burgdorferi* sensu lato strains with impact for the development of serological assays and indicates lateral gene transfer of the *dbpA* gene. *Int J Med Microbiol*. 2006;296 Suppl 40:250–66.
  46. Vollmer SA, Bormane A, Dinnis RE, Seelig F, Dobson AD, Aanensen DM, et al. Host migration impacts on the phylogeography of Lyme Borreliosis spirochaete species in Europe. *Environ Microbiol*. 2011;13(1):184–92.
  47. Framson PE, Nittayajarn A, Merry J, Youngman P, Rubens CE. New genetic techniques for group B streptococci: high-efficiency transformation, maintenance of temperature-sensitive pWW01 plasmids, and mutagenesis with Tn917. *Appl Environ Microbiol*. 1997;63(9):3539–47.
  48. Liu D, Hollingshead S, Swiatlo E, Lawrence ML, Austin FW. Rapid identification of *Streptococcus pyogenes* with PCR primers from a putative transcriptional regulator gene. *Res Microbiol*. 2005;156(4):564–7.
  49. Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol*. 2011;7:539.
  50. Casjens SR, Mongodin EF, Qiu WG, Luft BJ, Schutzer SE, Gilcrease EB, et al. Genome stability of Lyme disease spirochetes: comparative genomics of *Borrelia burgdorferi* plasmids. *PLoS One*. 2012;7(3):e33280.
  51. Casjens S, Eggers C, Schwartz I. *Borrelia* genomics: chromosome, plasmids, bacteriophages and genetic variation. In: Samuels S, Radolf J, editors. *Borrelia: molecular biology, host interaction and pathogenesis*. Norwich: Horizon Scientific Press; 2010. p. 27–52.
  52. Casjens SR, Fraser-Liggett CM, Mongodin EF, Qiu WG, Dunn JJ, Luft BJ, et al. Whole genome sequence of an unusual *Borrelia burgdorferi* sensu lato isolate. *J Bacteriol*. 2011;193(6):1489–90.
  53. Margos G, Vollmer SA, Ogden NH, Fish D. Population genetics, taxonomy, phylogeny and evolution of *Borrelia burgdorferi* sensu lato. *Infect Genet Evol*. 2011;11(7):1545–63.
  54. Ivanova LB, Tomova A, Gonzalez-Acuna D, Murua R, Moreno CX, Hernandez C, et al. *Borrelia chilensis*, a new member of the *Borrelia burgdorferi* sensu lato complex that extends the range of this genospecies in the Southern Hemisphere. *Environ Microbiol*. 2014;16(4):1069–80.
  55. Wang D, Botkin DJ, Norris SJ. Characterization of the *vls* antigenic variation loci of the Lyme disease spirochaetes *Borrelia garinii* lp90 and *Borrelia afzelii* ACA1. *Mol Microbiol*. 2003;47(5):1407–17.