Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes

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N-terminal lipidation is a major mechanism by which bacteria can tether proteins to membranes and one which is of particular importance to Gram-positive bacteria due to the absence of a retentive outer membrane. Lipidation is directed by the presence of a cysteine-containing 'lipobox' within the lipoprotein signal peptide sequence and this feature has greatly facilitated the identification of putative lipoproteins by gene sequence analysis. The properties of lipoprotein signal peptides have been described previously by the Prosite pattern PS00013. Here, a dataset of 33 experimentally verified Gram-positive bacterial lipoproteins (excluding those from *Mollicutes*) has been identified by an extensive literature review. The signal peptide features of these lipoproteins have been analysed to create a refined pattern, G+LPP, which is more specific for the identification of Gram-positive bacterial lipoproteins. The ability of this pattern to identify probable lipoprotein sequences is demonstrated by a search of the genome of *Streptococcus pyogenes*, in comparison with sequences identified using PS00013. Greater discrimination against likely false-positives was evident from the use of G+LPP compared with PS00013. These data confirm the likely abundance of lipoproteins in Gram-positive bacterial genomes, with at least 25 probable lipoproteins identified in *S. pyogenes*

Keywords: *Streptococcus pyogenes*, *Bacillus subtilis*, genomics, YidC, exported proteins

INTRODUCTION

The bacterial cell envelope is of major significance as the focal point for interaction between the bacterium and its environment, notably the host in the case of bacterial pathogens. Bacterial cell envelope proteins thus carry out numerous important functions including roles in adhesion, nutrient acquisition and a variety of interactions with host defences. In Gram-positive bacteria the matrix of peptidoglycan and other wall polymers surrounding the cell is not an efficient barrier to the passage of small macromolecules, due both to its inherent structural permeability and to its turnover during normal cell growth. Indeed, peptidoglycan may be sufficiently permeable as to allow the passage of

globular proteins of up to ~50 kDa in size (Demchick & Koch, 1996; Dijkstra & Keck, 1996). Consequently, in the absence of a retentive outer membrane, Gram-positive bacteria have evolved distinct mechanisms for retaining proteins within their cell envelopes, including covalent linkage to the peptidoglycan and non-covalent binding to teichoic acids and other cell envelope polymers (Navarre & Schneewind, 1999; Cossart & Jonquieres, 2000; Janulczyk & Rasmussen, 2001). Also significant among these mechanisms is membrane-anchoring of bacterial lipoproteins (Lpps) by covalent N-terminal lipidation (Braun & Wu, 1994; Sutcliffe & Russell, 1995). Lpps in Gram-positive bacteria perform important roles as substrate-binding proteins (SBPs) in ABC transporter systems; in antibiotic resistance; in cell signalling; in protein export and folding; in sporulation and germination; in conjugation and various other functions (Sutcliffe & Russell, 1995). Many of these Lpps, most notably the SBPs, perform functions equi-

Abbreviations: Lpp, lipoprotein; MSD, membrane-spanning domain; SBP, substrate-binding protein.

Fig. 1. Lipoprotein biosynthesis in Gram-positive bacteria. Lipid modification of prolipoproteins (by thioether linkage of diglyceride to the lipobox cysteine) is followed by signal peptide cleavage, generating the mature lipid-anchored lipoprotein. A possible further step whereby an additional fatty acid is amide-linked to the free amino terminus does not appear to occur in the low G+C Gram-positive bacteria and is not shown. The representative signal sequence shown is that of *S. mutans* MsmE (Sutcliffe *et al.*, 1993) where Cys₂₃ becomes Cys₁ of the mature Lpp. Charged amino acids of the signal peptide n-region are highlighted in bold.

valent to those carried out by periplasmic proteins of Gram-negative bacteria.

The archetypal bacterial Lpp is the murein lipoprotein of *Escherichia coli* characterized by Braun and co-workers (reviewed in Braun & Wu, 1994). Structural studies revealed that a diacylglycerol moiety is thioether linked to an N-terminal cysteine of this Lpp and that this lipid group serves to orientate the protein by anchoring it to the inner leaflet of the outer membrane. Chemically identical lipid modifications have since been proposed, primarily on the basis of protein sequence analysis (see below), for a great many proteins in both Gram-positive and Gram-negative bacteria, although relatively few have received extensive biochemical characterization.

Biosynthesis of bacterial lipoprotein of the 'Braun' type proceeds via a well conserved pathway that is apparently unique to prokaryotes (Fig. 1). Following signal-peptide-directed export of the prolipoprotein (proLpp), the enzyme prolipoprotein diacylglycerol transferase (Lgt) uses phospholipid substrates and catalyses the addition of a diacylglycerol lipid unit onto the thiol of a crucial conserved cysteine which is located within a 'lipobox'

motif at the cleavage region of the proLpp signal peptide (Sankaran *et al.*, 1995; Qi *et al.*, 1995). Subsequently, the signal peptide is removed by a specific lipoprotein signal peptidase II (Lsp) enzyme which cleaves within the lipobox to release the lipidated cysteine as the N-terminus of the mature Lpp (Braun & Wu, 1994; Sankaran & Wu, 1995). These two steps have been confirmed to be necessary and sufficient for protein lipidation in Gram-positive bacteria (Zhao & Wu, 1992; Witke & Götz, 1995; Leskelä *et al.*, 1999; Tjalsma *et al.*, 1999a, b; Bengtsson *et al.*, 1999; Petit *et al.*, 2001). A third step, wherein the Lpp N terminus is further modified by addition of an amide-linked fatty acid, is apparently not conserved as homologues of the enzyme lipoprotein aminoacyl transferase are not found in the genomes of low G+C Gram-positive bacteria (Tjalsma *et al.*, 1999a and our unpublished observations).

Processing of bacterial Lpp is thus dependent on both signal peptide features (directing protein export) and an appropriately placed lipobox (for prolipoprotein recognition and modification by Lgt and Lsp). The features of typical Lpp signal peptides are well established and consist of an n-domain (containing the charged amino acids lysine and/or arginine), a central hydrophobic h-domain and a cleavage c-region (Klein *et al.*, 1988; von Heijne, 1989; Braun & Wu, 1994; Tjalsma *et al.*, 2000). Sequence analysis of both Gram-negative and Gram-positive Lpp signal peptides suggests these are typically shorter than those of secretory signal peptides and this mainly reflects the finding that their c-regions are shorter and typically contain apolar amino acids. As such, the c-region is effectively a continuation of the h-domain that is distinguished primarily by the sequence conservation preceding the invariant lipid-modified cysteine (von Heijne, 1989; Braun & Wu, 1994). It is this sequence, typically Leucine₋₃-Alanine/Serine₋₂-Glycine/Alanine₋₁-Cysteine₊₁ at positions -3 to +1 that is referred to as the lipobox sequence and the consensus pattern of amino acid usage has been documented in PROSITE under the pattern entry PS00013/PDOC00013 (<http://www.expasy.ch/prosite/>). The Prosite consensus pattern syntax (single letter amino acid code) describes the sequence motif determining lipidation as {DERK}(6)-[LIVFWSTAG](2)-[LIVMFYSTAGCQ]-[AGS]-C wherein the permitted lipobox amino acids preceding the cysteine at positions -1 to -4 are indicated and the absence of charged residues (i.e. no D, E, R or K) within the signal peptide h-region is prescribed. Furthermore, the pattern is subject to the application of additional rules: firstly, the cysteine must be between positions 15 and 35 of the sequence under consideration and secondly, there must be at least one lysine or one arginine in the first seven positions of the sequence. These rules localize the pattern to N-terminal sequences with n-regions characteristic of signal peptides.

Molecular genetic studies, and genome sequencing projects in particular, have identified a large number of putative Lpp genes on the basis of the presence of possible lipobox sequences at the N termini of translated

protein sequences. Indeed, putative Lpps may represent at least 0.5–8.0% of the bacterial proteome (Chambaud *et al.*, 1999; Tjalsma *et al.*, 1999a; Haake, 2000). However, it remains possible that a significant proportion of these putative Lpps are ‘false-positives’, misidentified due to the coincident presence of a cysteine within the signal sequences of exported proteins or proteins targeted for insertion into plasma membranes. Thus it is desirable to develop a method for more accurately assigning sequences as putative Lpps. In this context it is significant that the lipobox amino acids are apparently subject to subtle taxon-specific restrictions. For example, the lipobox consensus defined from a dataset of 26 proven spirochaetal Lpps is distinctive in comparison to that of *E. coli* Lpps (Haake, 2000). Likewise, the lipobox sequences of a subset of Gram-positive bacterial Lpp (proven and putative) were more restrictive than that described in the PS00013 pattern at the –3 and –2 positions (Sutcliffe & Russell, 1995). To investigate this further we have defined a dataset of Gram-positive bacterial Lpps for which there is experimental evidence to support lipidation, and performed sequence analyses to create a revised pattern for the identification of Gram-positive bacterial putative Lpps. This pattern has been applied to the genome of the human pathogen *Streptococcus pyogenes*, for which a novel inventory of putative Lpps is presented herein.

METHODS

Construction of a dataset of experimentally verified Gram-positive bacterial Lpps. Several criteria were considered as evidence that a protein is lipidated *in vivo*. These included (1) metabolic labelling of the protein, in its source organism, with exogenously supplied radiolabelled fatty acid (normally palmitate, incorporation of which is typically detected by autoradiography following protein electrophoresis). This criterion excluded evidence of lipid radiolabelling of the protein following cloning into a heterologous Gram-negative bacterial host (usually *E. coli*) where the specificities of the Lpp processing enzymes may be subtly different. One exception within this criterion is the Cu,Zn superoxide dismutase Lpp of *Mycobacterium tuberculosis* where non-denaturing electrophoresis indicated that the native membrane-associated enzyme activity is electrophoretically identical to that which has been radiolabelled with palmitate upon expression in *E. coli* (D’Orazio *et al.*, 2001). (2) Inhibition of Lpp signal peptide processing by treatment with the antibiotic globomycin, which specifically inhibits Lsp (Inukai *et al.*, 1978). (3) Chemical characterization of the purified protein. (4) Evidence that protein processing is disrupted by mutation in either Lgt or Lsp, or following site directed mutagenesis to replace the lipobox cysteine. Evidence that proteins remain detergent-associated on phase partitioning with Triton X-114 was not included as this may not adequately discriminate Lpp from peripheral membrane proteins or proteins tethered to membranes by other mechanisms. Furthermore, although Lpps are apparently highly abundant in *Mollicutes* (Razin *et al.*, 1998; Chambaud *et al.*, 1999), these Lpps were excluded from the present analysis as it has been previously noted that they may have distinctive signal peptide features in terms of both length (Edman *et al.*, 1999) and their lipobox cleavage sites (Cleavinger *et al.*, 1994, 1995). In this respect it is also notable that the putative Lgt sequences in the *Mycoplasma* genomes

have distinctive sequence features (our unpublished observations). Moreover, variations in susceptibility to globomycin and alternative forms of lipidation were apparent in *Acholeplasma laidlawii* (Nyström *et al.*, 1986; Nyström & Wieslander, 1992). It was thus considered that these Lpps represent an abundant, distinctive subset that should be considered separately to avoid artificially polarizing the dataset. Following an extensive review of the available literature, 33 proteins that satisfied at least one of the above described criteria were identified as proven Lpps (Table 1).

Identification of Lpp sequences by pattern searching. Pattern documentation for the prokaryotic membrane lipoprotein lipid attachment site (PS00013) was obtained from the Prosite website (Falquet *et al.*, 2002; <http://www.expasy.ch/prosite/>). Pattern searches of the SWISS-PROT and TrEMBL databases were performed using the ScanProsite tool (<http://ca.expasy.org/tools/scanprosite/>), restricting the searches to entries specific for *Bacillus subtilis* or *S. pyogenes*. Protein sequences for further analyses were retrieved from SWISS-PROT and TrEMBL.

Protein primary sequence analyses. Membrane spanning domains (MSDs) in protein sequences were predicted using TMPred (Hofmann & Stoffel, 1993; http://www.ch.embl.net.org/software/TMPRED_form.html), set to a minimum length of 14 aa for the hydrophobic domains. Signal peptide analyses were performed using the refined hidden Markov model version 2.0 of SignalP (Nielsen *et al.*, 1997; Nielsen & Krogh, 1998; <http://www.cbs.dtu.dk/services/SignalP-2.0/>). Although Lpp signal peptide cleavage sites are not predicted, the graphical display from this service was particularly useful as it provided an indication of the lengths of predicted signal peptide n- and h-regions relative to the position of the possible lipobox cysteine. Where sequence features required further clarification, other methods for predicting transmembrane domains were also applied including TopPred2 (Claros & von Heijne, 1994; <http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html>) and DAS (Cserzo *et al.*, 1997; <http://www.sbc.su.se/~miklos/DAS/>).

Sequences homologous to the putative Lpps were identified by BLAST searches (Altschul *et al.*, 1997) using the National Center for Biotechnology Information tBLASTn server (<http://www.ncbi.nlm.nih.gov/BLAST/>), typically with the sequence filtering option switched off and the Expect value set at 0.001.

Access to sequence annotation of the *S. pyogenes* and *Streptococcus pneumoniae* genomes was obtained via the Entrez Genomes facility at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html>). Finally, other general searches were performed using the PEDANT genome sequence analysis tool (Frishman *et al.*, 2001; <http://pedant.gsf.de/>).

Criteria for exclusion of sequences as false-positives unlikely to encode Lpps. The N-terminal features of putative Lpps were individually analysed to determine whether they might be considered false-positives using the following criteria. Firstly, TMPred was used to determine the position of the most N-terminal MSD and also the number of additional MSDs. Those sequences where either an MSD was clearly absent or the most N-terminal MSD clearly extended beyond the lipobox cysteine (e.g. sequences likely to direct insertion of integral membrane proteins) were considered possible false-positives. The detection using TMPred of additional MSDs beyond the lipobox cysteine was not considered sufficient justification for excluding sequences, since both the CtaC and

Table 1. Gram-positive bacterial lipoproteins for which both sequence data and experimental evidence for lipidation is available

Categories for the experimental verification of protein lipidation are numbered the same as in Methods i.e. (1) metabolic labelling of the protein, in its source organism, with exogenously supplied radiolabelled fatty acid; (2) inhibition of Lpp signal peptide processing by treatment with the antibiotic globomycin, which specifically inhibits Lsp; (3) chemical characterization of the purified protein; (4) evidence that protein processing is disrupted by mutation of the biosynthetic enzymes Lgt or Lsp, or following site directed mutagenesis to replace the lipobox cysteine.

Source organism and protein	Experimental evidence				Reference
	1	2	3	4	
<i>Alicyclobacillus acidocaldarius</i> MalE	+				Hermann <i>et al.</i> (1996); Hülsmann <i>et al.</i> (2000)
<i>Bacillus cereus</i> BlaZ	+	+	+		Nielsen & Lampen (1982, 1983)
<i>Bacillus licheniformis</i> BlaP	+	+	+		Nielsen & Lampen (1982)
<i>Bacillus</i> sp. pS3 CccA			+		Fujiwara <i>et al.</i> (1993)
<i>B. subtilis</i> CtaC				+	Bengtsson <i>et al.</i> (1999)
<i>B. subtilis</i> KapB				+	Dartois <i>et al.</i> (1997)
<i>B. subtilis</i> OpuAC SBP		+		+	Kempf <i>et al.</i> (1997)
<i>B. subtilis</i> PrsA	+			+	Kontinen & Sarvas (1993); Tjalsma <i>et al.</i> (1999a)
<i>B. subtilis</i> PstS (YqgG)				+	Antelmann <i>et al.</i> (2001)
<i>B. subtilis</i> QoxA				+	Antelmann <i>et al.</i> (2001)
<i>Corynebacterium glutamicum</i> CtaC				+	Sakamoto <i>et al.</i> (2001)
<i>Helibacterium gestii</i> ChyA (c553)			+		Albert <i>et al.</i> (1998)
<i>Lactococcus lactis</i> PrtM	+			+	Haandrikman <i>et al.</i> (1991)
<i>Mycobacterium bovis</i> MPB83				+	Vosloo <i>et al.</i> (1997)
<i>Mycobacterium intracellulare</i> MI43	+				Nair <i>et al.</i> (1993)
<i>Mycobacterium tuberculosis</i> 19 kDa	+				Ashbridge <i>et al.</i> (1989); Young & Garbe (1991)
<i>M. tuberculosis</i> PhoS	+				Andersen & Hansen (1989); Young & Garbe (1991)
<i>M. tuberculosis</i> Cu,Zn SOD	+				D'Orazio <i>et al.</i> (2001)
<i>Staphylococcus aureus</i> Bla	+	+	+		Nielsen & Lampen (1982)
<i>Staphylococcus carnosus</i> ModA	+				Neubauer <i>et al.</i> (1999)
<i>Staphylococcus epidermidis</i> SitC	+				Cockayne <i>et al.</i> (1998)
<i>Streptococcus equi</i> HAP		+			Chanter <i>et al.</i> (1999); I. Sutcliffe & N. Chanter, unpublished
<i>Strep. equi</i> LppC		+			Hamilton <i>et al.</i> (2000)
<i>Strep. equi</i> MBL		+			Harrington <i>et al.</i> (2000)
<i>Streptococcus gordonii</i> SarA (HppA)	+				Jenkinson <i>et al.</i> (1996)
<i>Streptococcus mutans</i> MsmE	+	+			Sutcliffe <i>et al.</i> (1993)
<i>Streptococcus pneumoniae</i> AmiA	+				Alloing <i>et al.</i> (1994)
<i>Strep. pneumoniae</i> AliA	+				Alloing <i>et al.</i> (1994)
<i>Strep. pneumoniae</i> AliB	+				Alloing <i>et al.</i> (1994)
<i>Streptococcus pyogenes</i> LppC		+			Gase <i>et al.</i> (1997)
<i>Streptomyces chrysomallus</i> FKBP33	+				Pahl & Keller (1994)
<i>Streptomyces reticuli</i> CebE	+	+			Schlosser & Schremf (1996)
<i>Thermoanaerobium ethanolicus</i> XylF	+				Erbezniak <i>et al.</i> (1998a, b)

the QoxA proven Lpps have two additional MSDs predicted beyond their N-terminal lipid anchors (Bengtsson *et al.*, 1999; Sakamoto *et al.*, 2001; Antelmann *et al.*, 2001), as does the *Mycoplasma pneumoniae* F₀F₁ ATPase (Pyrowolakis *et al.*, 1998).

Sequences were also analysed using SignalP. Those sequences where an h-region was typically predicted to end within 2 aa of the lipobox cysteine were retained as possible Lpps.

Cumulatively, those sequences where signal peptide features were absent altogether and/or the lipobox cysteine was clearly internal rather than terminal to an h-region/MSD were considered to be likely false-positives. Those sequences where further clarification was needed were investigated using various other servers for predicting transmembrane regions (notably TopPred2 and DAS) and a consensus taken as to the position of the putative lipobox cysteine relative to the length of the first predicted MSD.

RESULTS AND DISCUSSION

Analysis of the signal peptide and lipobox features of experimentally verified Gram-positive bacterial Lpps

An extensive literature search identified 33 experimentally verified Lpps from Gram-positive bacteria (Table 1). Analysis of the lipobox features of these proteins (see Tables 2 and 3) confirmed previously observed preferences (Braun & Wu, 1994; Sutcliffe & Russell, 1995), notably in the high frequency of leucine at the -3 position, and alanine or serine at -2 of the lipobox. However, compared with the PS00013 pattern, some deviations and restrictions were apparent. Alanine and glycine were the only amino acids noted at the -1 position, as previously noted for proven Lpps generally (von Heijne, 1989). Significantly, it was observed that the *B. subtilis* KapB sequence contains a glutamic acid (E) at position -4 of its lipobox, an occurrence proscribed by the PS00013 pattern.

Analysis of this 33 Lpp dataset indicated that the n-regions had a mean length of 6.7 ± 3.5 and a range of 3–15 aa. Notably, two proven Lpps (KapB of *B. subtilis* and MI43 of *Mycobacterium intracellulare*) had N termini with no arginine or lysines in the first 7 aa, which is contradictory to the PS00013 additional rules. The mean h-region length was 12.1 ± 2.3 aa, with a range from 6–20 aa. These figures are in good agreement with the h-region features reported for the putative Lpp of *B. subtilis* (Tjalsma *et al.*, 2000). The mean cysteine position was 24.0 ± 3.6 (range 17–33) confirming that these signal peptides are typically shorter than those directing protein export in Gram-positive bacteria, for which a mean length of 32 has been reported (Nielsen *et al.*, 1997). It was also informative to consider the length of the membrane-spanning regions of these signal peptides i.e. the h-region plus the 5 typically apolar amino acids of the lipobox motif. This indicated the mean length of the combined h- and c-regions to be 17.1 aa, which is sufficient to span a typical bilayer membrane. These data suggest that the active site of the Lgt enzyme most likely interacts with the lipobox cysteine at the outer face of the cytoplasmic membrane. Sequence alignments of proven and putative Lgt enzymes from a wide range of taxa indicate that the most highly conserved residues are likely to be positioned at this interface (A. Hamilton, I. C. Sutcliffe. & D. J. Harrington, unpublished observations), as has been predicted for the critical aspartates in the active site of Lsp (Tjalsma *et al.*, 1999b).

Given that certain proven Lpps had signal peptide sequences features clearly contradictory to those described by the PS00013 pattern, and that additional discrimination against false-positives is likely to result from the derivation of a taxon-specific pattern, a modified pattern was defined for the proven Lpp signal peptide sequences of Gram-positive bacteria (Table 2). This pattern, $<[MV]-X(0,13)-[RK]-\{DERKQ\}(6,20)-[LIVMFESTAG]-[LVIAM]-[IVMSTAFG]-[AG]-C$ (using Prosite syntax), was termed G + LPP (Table 3).

To test the utility of this pattern, it was applied to a search of the genome of *B. subtilis*, as signal peptide dependent export has been extensively studied in this organism (Tjalsma *et al.*, 2000). A set of 103 putative Lpps of *B. subtilis* were identified by a pattern search with PS00013, whereas only 61 probable Lpps and the six proven Lpps of this organism were identified using the G + LPP pattern. The G + LPP pattern gave a greater discrimination (four rather than sixteen hits) against sequences considered false-positives using the criteria described herein (data not shown). Cumulatively, it was considered that the advantage of using the G + LPP pattern was the greater confidence that could be placed in the predictions that conforming sequences are indeed likely to be Lpps.

Application of the G + LPP pattern to analysis of the *S. pyogenes* genome

S. pyogenes sequences in the SWISS-PROT/TrEMBL database containing the PS00013 pattern were identified and compared to those identified in a similar pattern search with G + LPP (Table 4). The PS00013 search identified 36 sequences of which nine (25%) were excluded as unlikely Lpps (false-positives) using the criteria described herein. The G + LPP pattern search again proved more discriminatory than PS00013 in that 26 sequences were identified, of which only one (4%) was considered as an unlikely Lpp. Thus eight out of nine (89%) of the unlikely Lpps initially identified using PS00013 were excluded (Table 4). Both searches identified the only previously identified proven Lpp, the putative acid phosphatase LppC (Gase *et al.*, 1997; Malke, 1998). Similarly, both searches also identified the Lbp laminin-binding protein (Terao *et al.*, 2002) and three previously identified SBPs (Podbielski *et al.*, 1996; Podbielski & Leonard, 1998; Janulczyk *et al.*, 1999), all of which have previously been considered putative Lpps. Notably, however, the search with G + LPP identified an additional putative SBP sequence (Spy0903) that had been excluded using PS00013 on account of the extended n-region features of this signal sequence.

The 24 probable Lpps identified represent $\sim 1.5\%$ of the *S. pyogenes* proteome. This is comparable to the identification of 36 putative Lpps ($\sim 1.6\%$ of the proteome) in the *S. pneumoniae* genome (Tettelin *et al.*, 2001). Indeed, many of the putative Lpps of *S. pyogenes* have a pneumococcal putative Lpp homologue (the on-line version of this paper at <http://mic.sgmjournals.org> contains a supplementary table showing the outputs from BLAST searches with the *S. pyogenes* putative Lpp sequences) and $\sim 60\%$ have a putative Lpp homologue in the *B. subtilis* genome. As in *S. pneumoniae* and other Gram-positive bacteria, the majority of these probable Lpps are putative SBPs predicted to be involved in the transport of substrates such as amino acids/peptides, iron/ferrichromes, other metal ions, phosphate and sugars. The abundance of putative amino acids/peptide transporters has been noted previously (Ferretti *et al.*, 2001). One putative SBP, Spy1228, belongs to a recently

Table 2. Signal peptide features of the proven Gram-positive Lpp dataset

Signal peptide features have been separated to illustrate the charged n-region amino acids (bold), the hydrophobic h-region sequence and the lipobox preceding the lipidated cysteine.

Protein	n-region sequence (no. aa)	h-region sequence (no. aa)	Lipobox (c-region)					
			-4	-3	-2	-1	C _n [*]	+2
<i>A. acidocaldarius</i> MalE	VSVRR (5)	WGIVSTGVAALVLAGG (16)	A	I	A	G	C26	G
<i>Bacillus cereus</i> BlaZ	MFVLNKFSTNSHYKK (15)	IVPVLLSCA (10)	T	L	I	G	C30	S
<i>B. licheniformis</i> BlaP	MKLWFSTLKLKK (12)	AAAVLLFSCV (10)	A	L	A	G	C27	A
<i>Bacillus</i> sp. pS3 CccA	MKWK (4)	LAAMFLGVSL (10)	A	L	A	A	C19	G
<i>B. subtilis</i> CtaC	MVKHWR (6)	LILLALVPL (10)	L	L	S	G	C21	G
<i>B. subtilis</i> KapB	MSTFETGSIVKGFYK (15)	TGVYIG (6)	E	I	T	A	C26	R
<i>B. subtilis</i> OpuAC SBP	MLKK (4)	IIGIGVSAMLAL (12)	S	L	A	A	C21	G
<i>B. subtilis</i> PrsA	MKK (3)	IAIAAITATSIL (12)	A	L	S	A	C20	S
<i>B. subtilis</i> PstS	MKKNK (5)	LVLMLMAAFMM (12)	I	A	A	A	C22	G
<i>B. subtilis</i> QoxA	MVIFLFRALK (10)	PLLVLALLTVVF (12)	V	L	G	G	C27	S
<i>Corynebacterium glutamicum</i> CtaC	MEQQNKRGGLK (12)	ALLGGVLGLGGL (12)	A	M	A	G	C29	E
<i>Heliobacterium gestii</i> ChyA	MKNLK (5)	LIGIAAVMGLSMV (13)	A	L	T	A	C23	N
<i>Lactococcus lactis</i> PrtM	MKKKMRLLK (8)	VLLASTATALL (11)	L	L	S	G	C24	Q
<i>Mycobacterium bovis</i> MPB83	MINVQAK (7)	PAAAASLAAIAIA (13)	F	L	A	G	C25	S
<i>Mycobacterium intracellulare</i> MI43	MSASCAVPRLTR (12)	FAVFAVAGATAL (12)	S	L	S	A	C29	G
<i>Mycobacterium tuberculosis</i> 19 kDa	MKR (3)	GLTVAVAGAAILVA (14)	G	L	S	G	C22	S
<i>M. tuberculosis</i> PhoS	MKIR (4)	LHTLLAVLTAAPLLL (15)	A	A	A	G	C24	G
<i>M. tuberculosis</i> Cu,Zn SOD	MPKPADHR (8)	NHAAVSTSVLSALFLGAGAA (20)	L	L	S	A	C33	S
<i>Staphylococcus epidermidis</i> SitC	MKK (3)	ILALIAFLI (10)	I	L	A	A	C18	G
<i>Staphylococcus aureus</i> Bla	MKK (3)	LIFLIVIAL (9)	V	L	S	A	C17	N
<i>Staphylococcus carnosus</i> ModA	MKIK (4)	HFLIALVAICL (11)	V	L	A	G	C20	S
<i>Streptococcus equi</i> HAP	MTVLGTKACKR (11)	LGLAAVTLASVA (12)	A	L	M	A	C28	G
<i>Streptococcus equi</i> LppC	MTSKK (5)	ILNLVSLGLSLV (12)	L	I	F	G	C22	T
<i>Streptococcus equi</i> MBL	MKKK (4)	LSLALSAFLAVF (12)	M	L	V	A	C21	S
<i>Streptococcus gordonii</i> SarA (HppA)	MKKGK (5)	ILALAGVALLATG (13)	V	L	A	A	C23	S
<i>Streptococcus mutans</i> MsmE	MKWYKK (6)	IGLLGIVGLTSV (12)	L	L	A	A	C23	N
<i>Streptococcus pneumoniae</i> AmiA	MKKNR (5)	VFATAGLVLLAAG (13)	V	L	A	A	C23	S
<i>Strep. pneumoniae</i> AliA	MKSSK (5)	LLALAGVTLLAAT (13)	T	L	A	A	C23	S
<i>Strep. pneumoniae</i> AliB	MKKSKSK (7)	YLTLAGLVLTGTGV (13)	L	L	S	A	C25	G
<i>Streptococcus pyogenes</i> LppC SPy1882	MKSKK (5)	VVSVISLTLSLF (12)	L	V	T	G	C22	A
<i>Streptococcus chrysomallus</i> FKBP33	VRR (3)	IAGLLVAPLLLL (12)	S	A	V	A	C20	G
<i>Streptococcus reticuli</i> CebE	MRTSIRRSQR (10)	LMALAAVAALTTG (13)	L	L	A	G	C28	A
<i>Thermoanaerobium ethanolicus</i> XylF	MFKKVSKK (7)	FALLIVAMFTLGI (13)	I	L	S	G	C26	A

* No. of the lipidated cysteine.

recognized family of lipoproteins (Rosati *et al.*, 1999) which also includes the TcsA CD4+ T cell-stimulating antigen of *Listeria monocytogenes* (Campbell & Shastri, 1998). A further *S. pyogenes* putative SBP Lpp belonging

to this family (Spy0163, 65% identical to Spy1228) was identified as a 'false-negative' (see below). Whilst Spy1228 (like its pneumococcal homologue SP0845) is adjacent to other components of a putative ABC sugar

Table 3. Summarized signal peptide features, lipobox amino acid frequencies for 33 proven Gram-positive bacterial Lpps and pattern comparison with Prosite PS00013

The frequency of amino acid usage in the lipobox region is indicated and the deduced patterns (converted to Prosite syntax) for each signal peptide region are shown in comparison to the appropriate part of the PS00013 pattern.

Summary features	n-region	h-region	−4	−3	−2	−1	+1	+2
<i>n</i> = 33	M or V to start		A 0.27	L 0.76	A 0.45	A 0.55	C	S 0.33
	Mean 6.7 ± 3.5	Mean 12.1 ± 2.3	L 0.24	I 0.09	S 0.27	G 0.45		G 0.33
	Range 3–15	Range 6–20	V 0.15	A 0.09	T 0.09			A 0.12
	Last aa K or R	No DERKQ	S 0.09	V 0.03	V 0.06			N 0.09
			T 0.06	M 0.03	G 0.03			E 0.03
			I 0.06		I 0.03			R 0.03
			E 0.03		M 0.03			Q 0.03
			F 0.03		F 0.03			T 0.03
			G 0.03					
			M 0.03					
G + LPP pattern	<[MV]-X(0,13)-[RK]-	{DERKQ}(6,20)-	[LIVMFESTAG]-	[LVIAM]	[IVMSTAFG]-	[AG]-	C	
Prosite PS00013	Additional rule: at least	{DERK}(6)-	[LIVMFWSTAG](2)		[LIVMFYSTAGCQ]-	[AGS]-	C	
Additional rule: Cys between 15 and 35	one K/R in aa 1–7							

Table 4. Signal peptide analysis of *S. pyogenes* proteins identified by pattern searches with PS00013 and G + LPP

Sequence	Signal peptide features*	Cys no.	h-region†	Other MSDs‡	SignalP§	G + LPP	Verdict
Spy1882	MKS KK VVSISLTLSLFLVTGCA	22	6–23	0	G21	+	Proven
OppA	MKS SK WLAASVAILSVSALAACG	23	6–24	0	G24	+	Probable
Spy0210	MGVMM KQ KI KILTVIGLMTVGMSACH	25	11–26	0	C25	+	Probable
Spy0247	MRK VL R V KK NI KI ARIVPLVLLLVACG	26	16–34	4	G27	+	Probable
Spy0252	MNM KK LASLAMLGASVLGLAACG	22	9–24	0	G23	+	Probable
Spy0317	MI KK QYLG R ATIALASTLVLAACG	23	6–26	0	G24	+	Probable
Spy0319	MSF KK WIGIVGIALASTVLVACS	22	6–21	0	C22	+	Probable
Spy0351	M K LTLN R ILFSGLALSILLTLTGCV	24	8–26	4	V25	+	Probable
Spy0385	M KK LTLTLTLCLTTITLIACG	20	4–21	0	G21	+	Probable
Spy0453	MG KR MSLILGAFLSVFLLVACS	21	7–22	0	S22	+	Probable
Spy0604	MN KRF K TGFLALVAMLLVACS	20	8–24	0	C20	+	Probable
Spy0857	MT KKK G K LVLISLFVLAACL	19	8–26	0	G21	+	Probable
Spy0903¶	MLYYSYLN K SVTLLSIGLASLLLAACA	32	18–33	0	A31	+	Probable
Spy1228	MN KK FIGLGLASVAVLSLAACG	21	5–22	0	G22	+	Probable
Spy1245	M K M KK FFLLSLLALSTFFLSACS	23	7–27	0	S25	+	Probable
Spy1274	MI I KKR TVAILAIASSFFLVACQ	22	9–25	0	C22	+	Probable
Spy1290	MT KK HLLTLLLSFFTSFLVACS	22	6–22	0	A21	+	Probable
Spy1294	M K SWQ K VIVGGASLTLASTLLVCG	24	7–24	0	C24	+	Probable
Spy1390	M K NSN K LIASVVTLASVMALAACQ	23	7–23	0	A22	+	Probable
Spy1405	ML R KKK TTIVGIIILGMMIVTLLVGCL	25	9–28	0	L26	+	Probable
Spy1558	M KK GLLVTTGLACLGLLTACS	20	4–20	1	S21	+	Probable
Spy1795	MI KR CK GIGLALMAFFLVACV	20	8–25	1	V21	+	Probable
Spy2000	MS K YL K YFSIITLFLTGLILVACQ	23	7–23	0	A22	+	Probable
Spy2007	M KK GFFLMAMVVSVMIAAGCD	20	3–20	0	C20	+	Probable
Spy2037	M K QMN K LITGVVTLATVVTLSACQ	23	7–23	0	A22	+	Probable
Spy1361	M K T KK VIIIVGLLLSSQLTLIACQ	23	7–23	0	C23	–	Possible
Spy1972	M KKK VNQGS K RYQYLL KK WGIGFVIAATGTVVLGCT	35	19–36	1	C35	–	Possible
Spy2066#	MIN KK ISLGVLSILTAFSLQSVSYACT	26	6–32	0	S18	–	Possible
Spy0257	MTDLT K YQGIIPAFYACY	17	0	3	Absent	–	Unlikely
NanH							
Spy0422	MM KK PFYITTPIIYPSGKLHIGSAYTTIACD	30	0	3	Absent	–	Unlikely
MetS							
Spy0428	ML KKR YQLAIVLLLSCF	16	6–23	0	I20	–	Unlikely
Spy0456	MSM K FFEGLMSYHFLQNALITAVVIGIVSGAVGCF	34	18–38	6	Absent	–	Unlikely
MtsC							
Spy0764	MTNGL K YVLEQMLLLFIIAALACL	23	17–31	0	I28	–	Unlikely
EpuA							
Spy1094	MNN R H KRR Q KR KAFTLINLILFACI	25	13–36	0	F33	+	Unlikely
Spy1150	MS K IVVVGANHAGTACI	16	0	2	Absent	–	Unlikely
Nox							
Spy1209	M KR IVVANDLVGV G KVALSASIPLMASCL	28	10–30	1	Absent	–	Unlikely
PdxK							
Spy1593	ME KKKK VE K VNV R TFD R KTNAIFNILIGLFAISCI	34	22–44	5	F40	–	Unlikely
permease							

* n-region lysine and arginines are highlighted in bold.

† The position of the most N-terminal MSD identified using TMpred was determined.

‡ Total number of other MSDs predicted by TMpred.

§ Amino acid position predicted to represent the end of the n/h region using SignalP.

|| + indicates sequences also identified by pattern searching with G + LPP.

¶ Putative SBP sequence identified on searching with G + LPP but not by PS00013.

The signal sequence features of this sequence are unclear.

transporter in the *S. pyogenes* genome, Spy0163 is not (Ferretti *et al.*, 2001).

A second major functional category of *S. pyogenes* Lpps were those that may be involved in protein export and extracytoplasmic folding. Thus two homologues (Spy0247 and Spy0351) were identified of *B. subtilis* SpoIIIJ (Errington *et al.*, 1992), a probable Lpp that has recently been suggested to be associated with the export and processing of integral membrane proteins (Tjalsma *et al.*, 2000; Lührink *et al.*, 2001; Yen *et al.*, 2001). Both Spy0247 and Spy0351 are predicted to encode proteins with multiple MSDs in addition to the predicted N-terminal lipid anchor, as in SpoIIIJ (Lührink *et al.*, 2001; Yen *et al.*, 2001). Likewise, two homologous putative Lpps of the SpoIIIJ family have been identified in the pneumococcal genome (Tettelin *et al.*, 2001). The functional significance of the probable N-terminal lipidation of these SpoIIIJ family proteins is difficult to predict given that they are also integral membrane proteins. However, the lipid anchor may serve to correctly orientate an N-terminal domain and it is noted that several proven Lpps also have additional MSDs (see Methods).

In addition to the above, at least two probable Lpps (Spy1390 and Spy2037) may function as extracytoplasmic folding catalysts in *S. pyogenes*. Both proteins are homologous to the recently described pneumococcal putative maturase PpmA (Overweg *et al.*, 2000a, b) and to the proven Lpps PrsA of *B. subtilis* and PrtM of *Lactococcus lactis* (Haandrikman *et al.*, 1991; Kontinen & Sarvas, 1993). Collectively these proteins belong to the type C family of peptidyl-prolyl isomerases (Rudd *et al.*, 1995) which appear to direct correct protein folding. Thus these Lpps may act as folding catalysts or chaperones during protein export, although it is not clear whether this function is likely to be general or linked to the export of specific proteins. Since chaperones are likely to be required to interact with their cognate proteins as they emerge from the cytoplasmic membrane, the predicted localization of Lpps immediately adjacent to the external face of the membrane is consistent with this role. A further putative Lpp, peptidyl-prolyl isomerase, is discussed below.

Spy1558 is a member of the family of thioredoxin proteins and two homologous thioredoxin family members were identified in the pneumococcal genome (Tettelin *et al.*, 2001). Both Spy1558 and its pneumococcal putative Lpp homologue SP0659 are situated adjacent to genes encoding putative exported peptide methionine sulfoxide reductases. These enzymes repair the damage to proteins that results from the oxidation of methionine residues to methionine sulfoxide and this catalytic activity requires an associated thioredoxin regenerating system (Lowther *et al.*, 2000). This may therefore be the role of the adjacent Lpp.

In addition to Lpps belonging to the functional categories described above, three surface enzymes (LppC acid phosphatase; Spy0210, which contains a domain suggesting it may be a protease of the transglutaminase

family; and Spy0857, a putative peptidoglycan hydrolase), two conserved hypothetical proteins and an ORF with no significant homologues were identified as probable Lpps.

Three putative Lpp sequences were identified that conformed with the PS00013 pattern but not the G + LPP pattern and, as such, warrant further consideration. Of these, the putative pullulanase Spy1972 is unusual in the length of its signal sequence n-region and also contains a C-terminal LPXTG motif that may provide an alternative wall-anchoring mechanism (Janulczyk & Rasmussen, 2001). The signal peptide features of Spy1361 were consistent with an h-region preceding the possible lipobox but this sequence was excluded by G + LPP due to the presence of a glutamine residue within the h-region. Spy1361 was noted to contain a 300 aa domain homologous to the leucine-rich repeat region of *L. monocytogenes* internalin A that is preceded by an N-terminal domain containing four histidine-triad motifs (HXXHH) which is homologous to PhtE of *S. pneumoniae* (Adamou *et al.*, 2001). The signal peptide features of the third protein Spy2066, a putative dipeptidase, are ambiguous. Thus without further evidence for the Lpp nature of these proteins, it is prudent to consider these three proteins as possible rather than probable Lpps.

Possible 'false-negatives' not identified by pattern searching the *S. pyogenes* genome

A combination of strategies was used to locate possible Lpp sequences missed using either of the above pattern searches (i.e. possible 'false-negatives'). These included analyses of the *S. pyogenes* genome annotation, searches for homologues of pneumococcal Lpps, searches through PEDANT and very low stringency BLAST searches ($E=100$) using a representative selection of the signal sequences for the probable Lpps identified herein (Table 4). These analyses identified six possible false-negatives that warrant further consideration.

Four additional SBPs that may be possible Lpps were identified. One such sequence was Spy0163 which, as noted above, is a paralogue of Spy1228. This sequence was revealed by BLAST searches with Spy1228 and the pneumococcal putative Lpp SP0845 but found to be annotated without five potential N-terminal amino acids, including an alternative start methionine and two lysines that would complete the signal peptide n-region. This revised signal peptide for Spy0163 matches the G + LPP pattern and both the sequence motifs identified by Rosati *et al.* (1999) are present. Secondly, Spy1592 was identified as a putative SBP. The signal sequence of this protein contains a lipobox sequence which was excluded by the pattern searches because of an asparagine at the -4 position. However, the corresponding ORF (99% amino acid identity) in the *S. pyogenes* Manfredo genome (http://www.sanger.ac.uk/Projects/S_pyogenes/) contains a serine at this position, suggesting this putative SBP may indeed be a Lpp in some strains. Likewise, the putative amino acid SBP

Spy0778 and Spy1306, a homologue of pneumococcal MalX, were excluded as their possible lipobox sequences contained proline in the -4 position. As this is incompatible with both PS00013 and the G+LPP patterns, these SBPs should not be considered to be Lpps until proven otherwise, particularly as some SBPs (including the Spy0713 AdcA metal binding protein homologue) are evidently not Lpps (Turner *et al.*, 1999; Claverys, 2001).

The *S. pyogenes* genome also contains an additional possible Lpp, peptidyl-prolyl isomerase of the cyclophilin family, Spy0457, which is highly homologous to the pneumococcal SP0771 putative Lpp. Again, this sequence was excluded by the pattern searches as the putative lipobox sequence contains an asparagine at the -4 position. As with Spy1592, the corresponding ORF (99% amino acid identity) in the *S. pyogenes* Manfredo genome contains the acceptable amino acid serine at this position and so this protein is likely to be a Lpp, at least in some strains of *S. pyogenes*. As discussed above, peptidyl-prolyl isomerase Lpp may assist in the folding of exported protein(s).

Finally, Spy2033 was identified as a possible false-negative. This sequence has been shown to contain a functional signal sequence directing either protein export or cell surface localization (Gibson & Caparon, 2002). The deposited Spy2033 sequence has an abnormally long 64 aa signal sequence. However, it is notable that the h-region ends in a putative lipobox cysteine which is preceded by sequence consistent with a canonical Lpp signal peptide (M₄₁KFKKVLVIPALAL-AATCFLTAC₆₃). The alternative start at M₄₁ is also consistent with sequence alignments of Spy2033 with its homologue, the *Streptococcus cristatus* putative Lpp TptA. Thus it may be that the annotation of this sequence needs verification.

Conclusions

It is evident from the data herein that the identification of putative Lpp genes has been greatly facilitated by computer-assisted sequence analysis methods. Pattern searches such as those described here are useful in application to the published bacterial genomes (and other deposited sequences) and, with appropriate software [e.g. MacVector (Janulczyk & Rasmussen, 2001)], can also be applied to the publicly available unfinished genome sequences. Searches with the PS00013 pattern are useful in identifying possible Lpps, although more extensive analysis (Table 4 and unpublished observations) suggests that a significant proportion of these are unlikely to be Lpps (i.e. they are false-positives). This lack of stringency associated with the PS00013 pattern was recognized during the annotation of the *S. pneumoniae* genome as putative Lpps were initially identified using PS00013, then the dataset refined using a novel hidden Markov model (Tettelin *et al.*, 2001). Moreover, the ISREC ProfileScan Server (http://hits.isb-sib.ch/cgi-bin/hits_motifscan) for pattern matching is noted to consider PS00013 as a low specificity 'pattern that

matches very frequently'. The present analysis demonstrates the benefits of employing a taxon-specific approach to increase the stringency of the pattern used for Lpp identification. The G+LPP pattern described herein allows probable Lpps of Gram-positive bacteria (excluding *Mollicutes*) to be identified with greater confidence. Moreover, the G+LPP pattern identifies proven and putative Lpp signal sequences that are not detected by searching with the PS00013 pattern. Application of the G+LPP pattern to the genomes of *S. pyogenes* and *B. subtilis* confirms that putative Lpps represent an abundant (at least ~1.5% of the proteome) class of cell envelope proteins. Functional predictions suggest that the Lpps of *S. pyogenes* may contribute to the virulence of this important pathogen and so these proteins may represent targets for novel therapeutic interventions. However, it must be emphasized that *in silico* analyses such as these are of importance primarily as a foundation for experimental analyses.

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