Lipoprotein signal peptidase of *Streptococcus suis* serotype 2

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This paper reports the complete coding sequence for a proliprotein signal peptidase (SP-ase) of *Streptococcus suis*, Lsp. This is believed to be the first SP-ase described for *S. suis*. SP-ase II is involved in the removal of the signal peptide from glyceride-modified prolipoproteins. By using *in vitro* transcription/translation systems, it was shown that the *lsp* gene was transcribed *in vitro*. Functionality of Lsp in *Escherichia coli* was demonstrated by using an *in vitro* globomycin resistance assay, to show that expression of Lsp in *E. coli* increased the globomycin resistance. An isogenic mutant of *S. suis* serotype 2 unable to produce Lsp was constructed and shown to process lipoproteins incorrectly, including an *S. suis* homologue of the pneumococcal PsaA lipoprotein. Five piglets were inoculated with a mixture of both strains in an experimental infection, to determine the virulence of the mutant strain relative to that of the wild-type strain in a competitive challenge experiment. The data showed that both strains were equally virulent, indicating that the knockout mutant of *lsp* is not attenuated *in vivo*.

INTRODUCTION

Streptococcus suis is a Gram-positive organism that is an important cause of meningitis, arthritis and septicaemia in piglets. Piglets often do not survive the disease (Clifton-Hadley, 1983; Vecht *et al.*, 1985). Sows are symptomless carriers of *S. suis* on their tonsils and pass the bacteria on to their piglets, which are sensitive to the infection. Occasionally, humans get infected by *S. suis* (Arends & Zanen, 1988). Control of the disease is hampered by the lack of effective vaccines and sufficient, sensitive diagnostic tools. PCR assays have been developed for the detection of serotypes 1, 2, 7 and 9 (Smith *et al.*, 1999b; Wisselink *et al.*, 1999, 2002). For other serotypes no sensitive diagnostics are available yet. For development of vaccines and diagnostics, more insight into the process of pathogenesis will be very helpful. Virulence factors of *S. suis* are attractive vaccine candidates and can potentially be used as tools for detection of virulent serotypes of *S. suis*. Recently, an *in vivo* expression system was used to identify environmentally regulated promoters under different selection conditions (Smith *et al.*, 2001). With this system, 36 environmentally regulated genes were identified.

One of these genes, *ivs-23/iri-24* (Smith *et al.*, 2001) showed similarity in the database to the regulatory genes *cpsY* of *Streptococcus agalactiae* (Koskiniemi *et al.*, 1998) and *oxyR* of *Escherichia coli* (Demple, 1999). In *S. agalactiae*, a putative regulatory function on capsule expression was attributed to *cpsY* (Koskiniemi *et al.*, 1998). Because, in *S. suis*, *ivs-23/iri-24* is not linked to the capsule operon (Smith *et al.*, 1999a), we here determined the gene sequences flanking *ivs-23/iri-24* in *S. suis*. In this paper we describe a putative operon expressing the transcriptional regulator and show that the operon contains a prolipoprotein signal peptidase. This type II signal peptidase of *S. suis* (*lsp*) was cloned and characterized. Functionality of Lsp was demonstrated in *E. coli*. An insertional knockout mutant of *lsp* was constructed and the role of the prolipoprotein signal peptidase in the pathogenesis of *S. suis* was studied by comparing wild-type and mutant strains in an experimental infection in piglets. The data show that the *lsp* mutant is not attenuated *in vivo*.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics*	Source/reference
E. coli		
XL-1 Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ (proAB lacIqZ)M15] Tn10 (Tet^R)	Stratagene
S. suis		
10	Virulent serotype 2 strain	Vecht et al. (1989)
10ΔLsp	Isogenic lsp mutant of strain 10	This work
Plasmids		
pGEM7Zf(+)	Replication functions of pUC, Amp^R	Promega
pKUN19	Replication functions of pUC, Amp^R	Konings et al. (1987)
pMR11	pKUN19 containing S. suis mrp gene	Smith et al. (1992)
pC194	Cm^R	Horinouchi & Weisblum (1982b)
pUK21	Replication functions of pUC	Laboratory collection
pUK21-Cm	pUK21 with a 900 bp EcoRI–HindIII fragment containing Cm^R preceded by the MRP promoter inserted in HindIII site	Laboratory collection
pE194	Em^R	Horinouchi & Weisblum (1982a)
pIVS-E	Replication functions of pWVO1, Spc^R, promoterless erm gene of pE194	Smith et al. (2001)
pIVS-23	pIVS-E containing a 850 bp insert showing homology to S. agalactiae CpsY and to E. coli OxyR	Smith et al. (2001)
pLSP-2	pGEM7Zf(+) containing 3 kb DraI–ClaI fragment of lsp	This work
pLSP-3	pLSP-2 in which a Cm resistance cassette preceded by the MRP-promoter was inserted into the unique StuI site	This work

*Tet^R tetracycline resistance; Amp^R ampicillin resistance; Cm^R chloramphenicol resistance; Em^R erythromycin resistance.

METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. S. suis strains were grown in Todd–Hewitt broth (code CM 189; Oxoid) and plated on Columbia blood base agar plates (code CM 331; Oxoid), containing 6 % (v/v) horse blood. E. coli strains were grown in Luria broth (Miller, 1972) and plated on Luria broth containing 1·5 % (w/v) agar. If required, antibiotics were added in the following concentrations: chloramphenicol (Roche), 3·4 μg ml^{-1} for S. suis and 10 μg ml^{-1} for E. coli; and ampicillin (Roche), 100 μg ml^{-1} for E. coli.

In vitro transcription/translation assay. This assay was performed using the Prokaryotic DNA-Directed Translation Kit (Amersham Life Science) according to the manufacturer's instructions. TRAN^{35}S-LABEL (containing 70 % L-[^{35}S]methionine) (43·5 GBq mmol^{-1}; ICN Biomedicals) was used to label de novo-synthesized proteins. After the in vitro transcription/translation, SDS-PAGE was performed with the NuPAGE system according to the manufacturer's instructions. Proteins were separated on a 4–12 % gradient gel in NuPAGE MES buffer. The gel was dried under vacuum and the signal was detected using a phosphor-imager (STORM, Molecular Dynamics).

Globomycin resistance assay. Stationary-phase E. coli cells were diluted 1:50 in Luria broth containing appropriate antibiotics and grown at 37 °C for 8 h. Ten microlitres of exponential-phase E. coli culture was used to inoculate 100 μl Luria broth containing various concentrations of globomycin (a generous gift of Dr Shunichi Miyakoshi, Sankyo Co. Ltd, Tokyo, Japan). The globomycin concentrations tested were 0, 10, 20, 40, 80, 160 and 320 μg ml^{-1}. The cells were allowed to grow for 16 h, and the OD$_{630}$ was measured in a spectrophotometer.

DNA manipulations and sequence analysis. Routine DNA manipulations were performed as described by Sambrook et al., 1989. DNA sequences were determined on a 373A DNA Sequencing System (Applied Biosystems). Samples were prepared by use of an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analysed using the Lasergene program (DNASTAR). The BLAST software tool (Altschul et al., 1997) was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank/EMBL databases using the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov/BLAST/), typically using the tBlastN option with sequence filtering switched off and the maximum Expect value set at 0·001. Profile scanning for significant matches to database motifs was performed using the on-line ISREC ProfileScan Server (http://hits.isb-sib.ch/cgi-bin/hits_motifscan).

Southern blotting and hybridization. Chromosomal DNA was isolated as described by Sambrook et al. (1989). After digestion with restriction enzymes, DNA fragments were separated on 0·8 % (w/v) agarose gels and transferred to GeneScreen Plus hybridization transfer membrane (NEN Life Science Products) as described by Sambrook et al. (1989). DNA probes for the lsp and chloramphenicol resistance genes were labelled with [α-^{32}P]dCTP (111 GBq mmol^{-1}; Amersham Life Science) by use of a random-primed DNA labelling kit (Boehringer). The DNA on the blots was pre-hybridized at 65 °C for at least 30 min and subsequently hybridized at 65 °C for 16 h with the appropriate DNA probes in a buffer containing 0·5 M sodium phosphate, pH 7·2, 1 mM EDTA and 7 % SDS. After hybridization, the membranes were washed twice with a buffer containing

40 mM sodium phosphate, pH 7·2, 1 mM EDTA and 5 % SDS at 65 °C for 30 min and twice with a buffer containing 40 mM sodium phosphate, pH 7·2, 1 mM EDTA and 1 % SDS at 65 °C for 30 min. The signal was detected using a phosphor-imager (Storm, Molecular Dynamics).

Construction of a *lsp* knockout mutant. To construct the mutant strain 10ΔLsp, the pathogenic strain 10 (Vecht *et al.*, 1989, 1992) of *S. suis* serotype 2 was electrotransformed (Smith *et al.*, 1995) with the plasmid pLSP-3 (Fig. 1). In this plasmid the *lsp* gene was inactivated by the insertion of a resistance cassette, consisting of the chloramphenicol resistance gene from pC194 (Horinouchi & Weisblum, 1982b), preceded by the promoter region of the *mrp* gene (Smith *et al.*, 1992). To create pLSP-3, pLSP2 (Fig. 1) was digested with *Stu*I, and ligated to the 900 bp *Sma*I–*Eco*RV fragment from pUK21-Cm containing the chloramphenicol resistance cassette preceded by the promoter region of the *mrp* gene. The ligation mixture was transformed to *E. coli* XL-1blue cells, and ampicillin- and chloramphenicol-resistant colonies were selected on Luria broth containing 1·5 % (w/v) agar, 100 μg ampicillin ml^{-1} and 10 μg chloramphenicol ml^{-1}. After electrotransformation of strain 10 with pLSP-3, chloramphenicol-resistant colonies were selected on Columbia blood base agar plates containing 3·4 μg chloramphenicol ml^{-1}. Southern blotting and hybridization experiments were used to screen for double-crossover integration events (data not shown).

Phenotypic characterization of the *lsp* knockout mutant. *S. suis* strains S10 and 10ΔLsp were grown in the presence and absence of [U-^{14}C]palmitate (Amersham Bioscience) to allow radiolabelling of lipoproteins. Overnight cultures of *S. suis* S10 and 10ΔLsp were diluted 1:20 into 5 ml pre-warmed Todd–Hewitt broth in triplicate. They were incubated at 37 °C for 1–2 h (early exponential phase), before the addition of either [^{14}C]palmitate (25 μCi [925 kBq] in 137 μl ethanol) or ethanol (137 μl) as controls. Control broths were used to monitor growth (OD$_{595}$) until late-exponential phase. Cells from the cultures containing radiolabel

were harvested by microcentrifugation (13 000 **g** for 5 min), resuspended in 1 ml sterile phosphate-buffered saline and recentrifuged. This was repeated three times and the washed cells were then prepared for SDS-PAGE analysis as described previously (Hamilton *et al.*, 2000). After drying, the gels were exposed to Kodak BIOMAX MR2 film (Anachem) at −80 °C for about 14 days. Autoradiograms were developed using Kodak GBX developer and fixer (Sigma) according to the manufacturer's instructions.

Western blotting following SDS-PAGE was performed as described previously (Hamilton *et al.*, 2000) using a rabbit polyclonal antiserum raised against the PsaA putative lipoprotein of *Streptococcus pneumoniae* (kindly supplied by Dr Jacquelyn Sampson, National Center for Infectious Diseases, Atlanta, GA, USA). This antiserum cross-reacts with PsaA and other lipoproteins in the streptococcal LraI family (Harrington *et al.*, 2000). BLAST searches of the draft *S. suis* genome, which has been made available by the Sanger Centre (http://www.sanger.ac.uk/Projects/S_suis/) suggested the presence of a PsaA homologue (LraI family member) in *S. suis* and that this sequence includes a signal peptide matching the consensus for proven Gram-positive bacterial lipoproteins (Sutcliffe & Harrington, 2002).

Experimental infections. Germfree piglets, crossbreeds of Great Yorkshire and Dutch Landrace, were obtained from sows by caesarean sections. The surgery was performed in sterile flexible film isolators. Piglets were allotted to groups of 5, and were housed in sterile stainless steel incubators. Housing conditions and feeding regimens were as described before (Vecht *et al.*, 1989, 1992). Six-day-old piglets were inoculated intranasally with about 10^7 c.f.u. of *Bordetella bronchiseptica* 92932, to predispose them to infection with *S. suis*. Two days later the piglets were inoculated intranasally with 10^6 c.f.u. of *S. suis* strain 10 plus 10^6 c.f.u. of *S. suis* strain 10ΔLsp. To determine differences in virulence between wild-type and mutant strains, LD$_{50}$ values should be determined. To do this, large numbers of piglets are required, which for ethical reasons is not acceptable. To circumvent this problem, we performed co-colonization studies. To monitor for the presence of *S. suis* and *B. bronchiseptica* and to check for absence of contaminants, swabs taken from the nasopharynx and the faeces were cultured three times a week. The swabs were plated directly onto Columbia agar containing 6 % horse blood, or grown for 48 h in Todd–Hewitt broth and subsequently again plated onto Columbia agar containing 6 % horse blood. Piglets were monitored twice a day for clinical signs and symptoms, such as fever, nervous signs and lameness. Blood samples from each piglet were collected three times a week. Leukocytes were counted with a cell counter. The piglets were killed when specific signs of an *S. suis* infection were observed, such as arthritis or meningitis, or when they became mortally ill. The other piglets were killed 2 weeks after inoculation with *S. suis*. All piglets were examined for pathological changes. Tissue specimens from heart, lung, liver, kidney, spleen and tonsil, and from the organs specifically involved in an *S. suis* infection (central nervous system, serosae and joints), were sliced with a scalpel or a tissuemiser. Tissue slices from each organ or site were resuspended in 2–25 ml Todd–Hewitt broth containing 15 % (v/v) glycerol, depending on the size of the tissue slice. The suspension was centrifuged at 3000 r.p.m. for 5 min. The supernatant was collected and serial dilutions were plated on Columbia agar containing 6 % horse blood, as well as on Columbia agar plates containing 6 % horse blood and 3·4 μg chloramphenicol ml^{-1} to quantitate the number of wild-type and mutant bacteria present. The number of mutant strain 10ΔLsp cells was determined by counting the number of c.f.u. on the appropriate serial dilution on the selective plates; the number of wild-type strain 10 cells was determined by counting the number of c.f.u. on the appropriate serial dilution on the Columbia agar blood plates, from which the number of c.f.u. counted on the selective plates was subtracted. When both wild-type and mutant bacteria were found in tissues, the ratio of wild-type and mutant
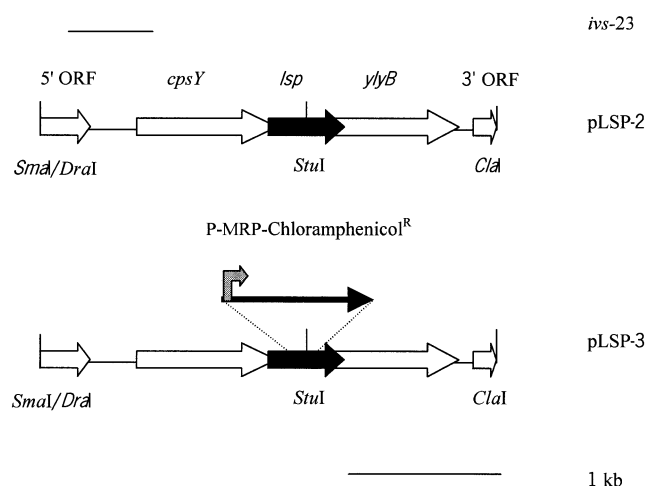
Fig. 1. Schematic representation of pLSP-2 and pLSP-3. A 3 kb *Cla*I–*Dra*I fragment that hybridized with probe *ivs-23/iri-24* was cloned into pGEM7Zf(+) digested with *Cla*I and *Sma*I, yielding pLSP-2. To create pLSP-3, the 900 bp *Sma*I–*Eco*RV fragment of pUK21-Cm, containing the chloramphenicol resistance gene from pC194 (Horinouchi & Weisblum, 1982b), preceded by the promoter region of the *mrp* gene (Smith *et al.*, 1992), was inserted in the *Stu*I site of *lsp*.

strain was determined more precisely, by toothpicking about 100 individual colonies onto both Columbia blood base agar plates and Columbia blood base agar plates containing 3·4 µg chloramphenicol ml^{-1}.

All animal experiments were approved by the ethical committee of the Institute for Animal Science and Health in accordance with the Dutch law on animal experiments.

RESULTS

lsp is located in a putative operon

One of the clones selected from a genomic library of *S. suis* serotype 2 was induced both *in vivo* and under iron-restricted conditions. This clone, *ivs-23/iri-24*, showed similarity in the database to the 5′ part of *cpsY* of *Streptococcus agalactiae*, and to *oxyR* of *E. coli* (Smith *et al.*, 2001). A ^{32}P-labelled probe of *ivs-23/iri-24* was used to identify the flanking regions from chromosomal DNA of *S. suis* strain 10. A 3 kb *Cla*I–*Dra*I fragment was detected and cloned into pGEM7Zf(+), yielding pLSP-2 (Fig. 1). The complete sequence of the insert was determined. Examination of the sequence revealed the presence of three complete and partially overlapping ORFs, and two incomplete ORFs (Fig. 1). The two incomplete ORFs are located at the 5′ and 3′ ends of the sequence and showed similarity in the database to genes encoding a regulatory protein for C-P lyase of *Streptomyces griseus* and to a gene encoding PHNA protein of *E. coli* respectively. 5′ of the first complete ORF, a putative −35 and −10 promoter sequence was found. Upstream of each start codon putative RBSs were found. 3′ of the third complete ORF, putative −35 and −10 promoter sequences were found. Together these data suggest that the three ORFs are transcribed as an operon and that the incomplete ORFs at the 3′ and 5′ end of the sequence do not belong to this operon.

The first ORF of the putative operon encoded a 304 amino acid protein that was highly similar (219/300 [73 %] amino acid identity) to *Streptococcus pneumoniae* SP0927, a putative transcriptional regulator of the LysR family, along with similarities to other regulators such as CpsY and OxyR (as described above). Sequence analysis confirmed that this translated sequence matched the database motifs for both the LysR family helix–turn–helix (Pfam PF00126) and substrate binding (Pfam PF03466) domains. The second ORF of the putative operon encoded a 154 amino acid protein that was highly similar (91/152 [59 %] amino acid identity) to *Streptococcus agalactiae* SAG1366 and to *Streptococcus pneumoniae* SP0928 (78/144 [54 %] amino acid identity), both of which are annotated as putative prolipoprotein signal peptidases (Lsp; type II signal peptidase). Sequence analysis confirmed that this translated sequence matched the database motif for the signal peptidase II family (Pfam PF01252). Finally, the last ORF of the putative operon encoded a 297 amino acid protein that was highly similar (254/297 [85 %] amino acid identity) to *Streptococcus pneumoniae* SP0929, a putative ribosomal

pseudouridine synthase. Sequence analysis confirmed that this translated sequence matched the database motifs for both an S4 domain (Pfam PF01479), which probably mediates binding to RNA, and the RluD pseudouridine synthase family (Pfam PF00849; PDOC00869). Pseudouridine synthases modify RNA base composition by converting uracil bases to pseudouridine (Conrad *et al.*, 1998). Inspection of the published microbial genomes and the publicly available unpublished genomes for *Streptococcus gordonii* and *Streptococcus mitis* indicated that this putative three-ORF operon is well conserved within the streptococcal genomes (e.g. the SP0927–SP0929 locus: Tettelin *et al.*, 2001). Moreover, chromosomal loci encoding putative Lsp and putative pseudouridine synthase enzymes are apparently uniformly present in the genomes of Gram-positive bacteria (e.g. LL0997 and LL0998 in the genome of *Lactococcus lactis*: Bolotin *et al.*, 2001) although this gene arrangement is not conserved in other taxa such as the Proteobacteria. The upstream presence of the putative LysR family member is not conserved in the non-streptococcal Gram-positive bacterial genomes and a variety of genes may be found preceding *lsp* (e.g. *L. lactis* LL0996 encodes a putative lumazine synthase). In this respect it is noted that in all the available streptococcal genomes there is sequence overlap between the ORFs encoding the putative Lsp and the pseudouridine synthase, whereas the overlap between *lysR* and *lsp* is not fully conserved.

Sequence analysis of Lsp of S. suis

A hydrophobicity plot (Kyte–Doolittle) and various tools for the prediction of membrane-spanning domains revealed the presence of four hydrophobic regions (data not shown), suggesting a similar membrane localization for *S. suis* Lsp as described for other Lsp enzymes (Witke & Götz, 1995; Prágai *et al.*, 1997; Tjalsma *et al.*, 1999b). The protein sequence of *S. suis* Lsp was compared to the sequences of other characterized signal peptidase II enzymes, notably that of *Bacillus subtilis*, wherein five conserved domains forming a potential active site have recently been identified (Zhao & Wu, 1992; Sankaran & Wu, 1994a; Witke & Götz, 1995; Prágai *et al.*, 1997; Tjalsma *et al.*, 1999b). This analysis confirmed that the strictly conserved N-terminal Domain I aspartic acid (D14 in the *B. subtilis* sequence) which is necessary for enzyme stability and function (Sankaran & Wu, 1994b; Tjalsma *et al.*, 1999b) is conserved in the Lsp sequence. The other five critical residues identified by Tjalsma *et al.* (1999b) are located in the two motifs NXXD (Domain III) and FNXAD (Domain V), wherein the two aspartates are proposed to form a catalytic dyad in the protease active site. All five of the critical residues identified in these two motifs are fully conserved in the *S. suis* Lsp sequence.

Lsp is expressed in vitro

Expression of Lsp was determined by using an *E. coli*-based *in vitro* transcription/translation system on pLSP-2. pGEM7Zf(+) was used as a negative control. Three

proteins were expressed that corresponded very well to the predicted sizes of the *S. suis* LysR family protein, the putative pseudouridine synthase and Lsp (respectively 34·7, 32·8 and 17·5 kDa) (data not shown). The negative control pGEM7Zf(+) expressed the α-LacZ fragment as well as β-lactamase (Amp) (data not shown). These data clearly showed that all three genes were expressed very efficiently from pLSP-2.

Globomycin resistance

To test whether Lsp was functional in *E. coli*, we used a globomycin sensitivity assay. Globomycin is a cyclic peptide antibiotic that specifically inhibits the processing of pro-lipoprotein to mature lipoprotein (Inukai *et al.*, 1978). Gram-negative organisms, like *E. coli*, are especially sensitive to this antibiotic due to inhibition of the murein lipoprotein processing. Overexpression of cloned signal peptidase genes from both Gram-negative and Gram-positive bacteria was shown to cause globomycin resistance in *E. coli* (Tokunaga *et al.*, 1983; Zhao & Wu, 1992; Witke & Götz, 1995). This globomycin resistance is generally used to demonstrate functionality of lipoprotein signal peptidases. pLSP-2 was used for the expression of Lsp. In pLSP-3, a chloramphenicol resistance cassette was inserted into the *lsp* gene to inactivate the gene. This construct was used as a negative control. As a second negative control, pGEM7Zf(+) without insert was included in the assay. *E. coli* cultures containing either one of the plasmids were grown overnight in media containing various concentrations of globomycin. After 16 h of growth the OD_{630} of the cultures was measured. Fig. 2 shows that *E. coli* harbouring pGEM7Zf(+) or pLSP-3 did not grow in the presence of a concentration of globomycin higher than 20–40 μg ml^{-1}. In contrast, *E. coli* harbouring pLSP-2 could grow in the presence of a concentration of globomycin of at least 320 μg ml^{-1} (Fig. 2), although the maximum optical density measured
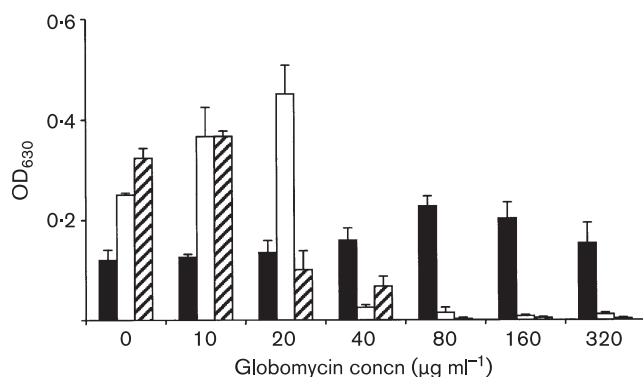
for this strain was lower than for the other strains. This indicates that the expression of Lsp and/or either of the other proteins encoded on pLSP-2 slightly inhibited the growth of *E. coli*. The increase of globomycin resistance due to expression of Lsp demonstrates that *lsp* indeed encodes a signal peptidase II that is functional in *E. coli*.

Role of Lsp in pathogenesis of *S. suis* serotype 2

To test the role of Lsp in the pathogenesis of *S. suis*, an isogenic knockout mutant of Lsp was constructed in strain 10, yielding strain 10ΔLsp. Strain 10ΔLsp was constructed by electroporation of pLSP-3, in which the *lsp* gene was inactivated by insertion of a chloramphenicol resistance cassette, into *S. suis* (Fig. 1) (Smith *et al.*, 1995). Southern blotting and hybridization experiments were used to discriminate isolates in which double-crossover events had occurred from those in which single-crossover events had occurred (data not shown). To characterize the mutant phenotype, the strain 10 parent and strain 10ΔLsp were grown in the presence of radiolabelled palmitic acid, and radiolabelled lipoproteins were visualized after SDS-PAGE and autoradiography (Fig. 3). The signal peptide processing
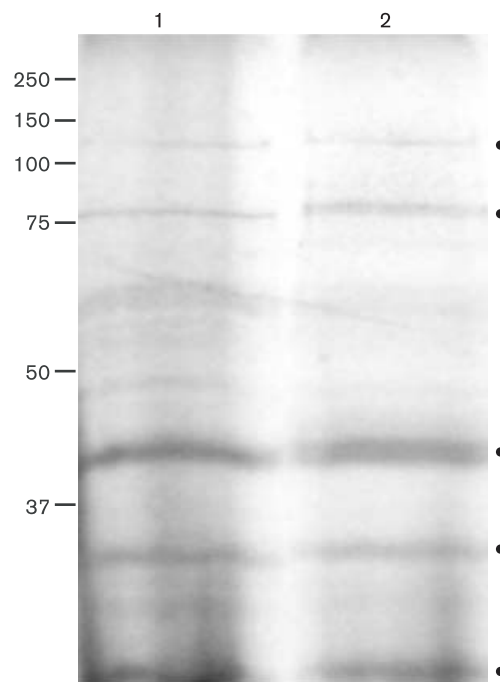
Fig. 3. Radiolabelling of *S. suis* lipoproteins with [U-^{14}C]palmitic acid. *S. suis* strains S10 and 10ΔLsp were grown in the presence of [U-^{14}C]palmitate and the labelled proteins were analysed by SDS-PAGE and autoradiography. Lane 1, *S. suis* strain S10; lane 2, *S. suis* strain 10ΔLsp. Positions of the molecular mass standards in kDa are marked on the left. Some of the radiolalled bands shifted to higher molecular masses (consistent with the accumulation of prolipoproteins with uncleaved signal peptides) are indicated on the right (●).
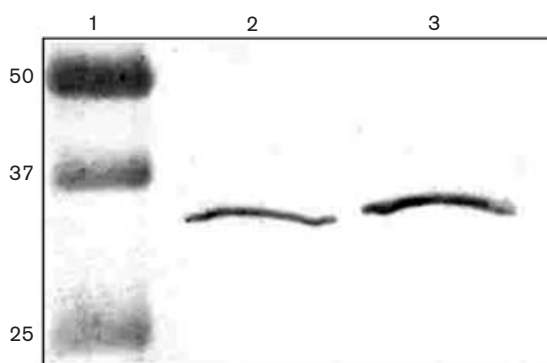

Fig. 2. Globomycin resistance assay. OD_{630} of *E. coli* cultures harbouring different plasmids was measured after 16 h of growth. Black bars, pLSP-2; white bars, pGEM7Zf(+); hatched bars, pLSP-3. Bars represent the means of triplicate experiments; error bars indicate the standard error of the mean (SEM).

of at least four radiolabelled proteins was affected in strain 10ΔLsp, as indicated by a shift of the radiolabelled protein band to a higher molecular mass, which is consistent with the accumulation of the prolipoprotein form (Fig. 3, lane 2). However, the appearance of doublets (prolipoprotein and putative 'mature-like' forms) suggested that some alternative path of lipoprotein processing was also active in the case of some lipoproteins. To verify the mutant phenotype further, Western blot analysis was performed using a polyclonal antibody to the pneumococcal PsaA lipoprotein. Consistent with the bioinformatic detection of a pneumococcal PsaA homologue (LraI family lipoprotein) in *S. suis* (see Methods), a cross-reacting band of about 35 kDa was detected in strain 10, whereas this band was shifted to a position consistent with the accumulation of the prolipoprotein in strain 10ΔLsp (Fig. 4). Cumulatively, these data confirmed that lipoprotein processing is disrupted in strain 10ΔLsp.

Because we were unable to determine LD_{50} values for the mutant in pigs for ethical reasons, it was decided to do a competitive co-colonization experiment with the wild-type strain. The virulence of strain 10ΔLsp was compared to that of the wild-type in an experimental infection in piglets. The mutant strain 10ΔLsp was mixed with wild-type bacteria in competition challenge experiments to determine the relative attenuation of the mutant strain. Using *in vitro* conditions, the growth rates of wild-type and mutant strain in Todd–Hewitt medium were found to be essentially identical (data not shown). Wild-type and mutant bacteria were inoculated at an actual ratio of 4:1 (1.5×10^6 c.f.u. of strain 10 and 4×10^5 c.f.u. of strain 10ΔLsp), in five piglets (nos 1, 26, 42, 44 and 49). During the experiment, piglets that developed

specific *S. suis* symptoms (meningitis, arthritis or mortal illness) were killed. Piglets that did not develop these symptoms were killed at the end of the experiment. Three out of five piglets developed central nervous signs (nos 26, 42 and 44); one of them also developed a severe pericarditis (no. 44). Piglet no. 49 developed a severe arthritis in one leg. Piglet no. 1 survived until the end of the experiment, but at the post-mortem section a peritonitis and probably a starting meningitis was observed. As shown in Fig. 5(a), wild-type and mutant bacteria were re-isolated from tonsils in a ratio of about 14:1 (wild-type:mutant), while the input ratio was 4:1. This indicated that the mutant bacteria colonized the tonsil less efficiently than the wild-type bacteria. Fig. 5(b) shows that no large differences were found in the ratio of re-isolated wild-type bacteria and mutant bacteria, nor in the number of organs that were colonized by wild-type or mutant bacteria. Taken together, these data indicate that, although the mutant seemed to colonize the tonsil slightly less efficient that the wild-type strain, the mutant strain 10ΔLsp is as virulent as the wild-type strain.

DISCUSSION

In this paper we describe the identification and characterization of Lsp, a lipoprotein signal peptidase of *S. suis* serotype 2. The *lsp* gene was found to be part of a putative operon containing three overlapping genes. These genes were preceded by a putative promoter sequence. Downstream of the third gene was another putative promoter sequence. These findings suggest that this putative operon
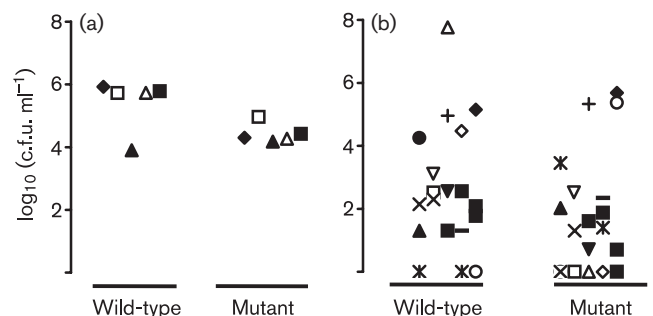

Fig. 4. Western blot analysis of the putative *S. suis* homologue of pneumococcal PsaA. Cell extracts of *S. suis* strains S10 and 10ΔLsp were analysed by SDS-PAGE and Western blotting using a polyclonal anti-PsaA antibody. Lane 1, Bio-Rad prestained standards with molecular masses in kDa marked on the left. Lane 2, *S. suis* strain S10. Lane 3, *S. suis* strain 10ΔLsp. The shifting to a higher molecular mass of the cross-reacting band in lane 3 is consistent with the accumulation of a prolipoprotein with an uncleaved signal peptide.

Fig. 5. Efficacy of colonization of wild-type and mutant bacteria on various organs of infected pigs. (a) Colonization of wild-type strain 10 and mutant strain 10ΔLsp on the tonsils: ◆, piglet no. 1; □, piglet no. 26; ▲, tonsil piglet no. 42; △, tonsil piglet no. 44; ■, tonsil piglet no. 49. (b) Colonization of the specific organs (CNS, central nervous system): ▲, CNS piglet no. 1; ■, joints piglet no. 1; ●, peritoneum piglet no. 1; □, spleen piglet no. 26; △, CNS piglet no. 26; _, kidney piglet no. 42; ◆, CNS piglet no. 42; ×, joints piglet no. 42; +, CNS piglet no. 44; ✳, joints piglet no. 44; ○, pericardium piglet no. 44; ▽, spleen piglet no. 49; ▼, heart piglet no. 49; ◇, joint piglet no. 49. Each symbol represents the numbers of wild-type or mutant bacteria isolated from one particular organ, from one pig.

contains only the three overlapping genes. Besides *lsp*, this putative operon contained a gene encoding a protein that is predicted to belong to the LysR family of transcriptional regulators and a gene encoding a putative pseudouridine synthase. This chromosomal locus was noted to be conserved in other streptococcal genomes, whilst the association of the *lsp* gene with that for a putative pseudouridine synthase is conserved in Gram-positive bacterial genomes generally. The LysR family transcriptional regulator was identified with an IVET system as being environmentally regulated (Smith *et al.*, 2001). Two different conditions were used to select environmentally regulated promoters: iron-restricted conditions and *in vivo* conditions. With both selections, the regulator gene was found. It is generally accepted that environmentally regulated genes, especially *in vivo*-induced genes, are potential virulence factors (Mahan *et al.*, 1993). For this reason we studied the role of the predicted Lsp enzyme encoded by the gene adjacent to the regulator in the pathogenesis of *S. suis* serotype 2.

Lsp is involved in the removal of the signal peptide from diacylglyceride-modified prolipoproteins (Sankaran & Wu, 1994a, b). In Gram-negative organisms several enzymes involved in the lipid-modification of prolipoproteins, including Lsp, are essential for normal growth, cell division and viability (Sankaran & Wu, 1994a, b; Gan *et al.*, 1993; Gupta *et al.*, 1993; Williams *et al.*, 1989; Yamagata *et al.*, 1982). In contrast, *lsp* of the Gram-positive organism *B. subtilis* is not necessary for growth and viability, even though the PrsA lipoprotein is an essential maturase enzyme (Tjalsma *et al.*, 1999a). Moreover, in *S. pneumoniae*, one of the enzymes involved in lipid-modification, prolipoprotein diacylglyceryl transferase (encoded by *lgt*), is not essential for cell growth *in vitro*, but is essential for viability during infection (Petit *et al.*, 2001). In Gram-positive organisms several roles have been attributed to lipoproteins such as participation in antibiotic resistance, ABC transporter systems, adhesion, protein export and extracytoplasmic folding, and sensory systems (Sutcliffe & Russell, 1995; Sutcliffe & Harrington, 2002). The abundance of putative lipoproteins in typical bacterial genomes (Sutcliffe & Harrington, 2002) suggests that this class of proteins is of considerable physiological significance. In streptococci other than *S. suis*, several lipoproteins have been shown to be involved in virulence (Burnette-Curley *et al.*, 1995; Berry & Paton, 1996; Kitten *et al.*, 2000; Brown *et al.*, 2001; Marra *et al.*, 2002). Moreover, mutants disrupted in *lsp* have been shown to be attenuated in signature-tagged mutagenesis studies of *Staphylococcus aureus* (Mei *et al.*, 1997; Coulter *et al.*, 1998).

Type II signal peptidase activity of Lsp was initially demonstrated using a globomycin assay. We showed that overexpression of Lsp in *E. coli* resulted in an increased resistance to globomycin. To study the role of Lsp in the pathogenesis of *S. suis*, an isogenic knockout mutant of *lsp* was constructed in a wild-type *S. suis* serotype 2 strain. To construct the mutant, we used a plasmid replicating in

E. coli in which the *lsp* gene was inactivated by the insertion of a chloramphenicol resistance gene (pLSP-3). In the globomycin assay, *E. coli* containing pLSP-3 did not show an increased globomycin resistance, whereas the construct containing the intact *lsp* gene did. This strongly indicated that in pLSP-3, Lsp was non-functional. To confirm the phenotype of strain 10ΔLsp, lipoproteins were radiolabelled by growth in the presence of radiolabelled [^{14}C]palmitic acid. Comparison with the lipoprotein profile for strain 10 indicated that multiple radiolabelled bands accumulated in a higher molecular mass form, consistent with a failure to remove signal peptides from prolipoproteins (Fig. 3). This observation was confirmed by Western blot analysis, which demonstrated the accumulation in strain 10ΔLsp of the prolipoprotein form of a putative LraI family member (PsaA homologue; Fig. 4).

The virulence of the *S. suis lsp* mutant was tested in an experimental infection model in piglets. Since we are unable to determine LD$_{50}$ values for the mutant strain in pigs, it was decided to compare the virulence of strain 10ΔLsp to the wild-type *S. suis* strain 10 in a competitive co-colonization assay in piglets. These kinds of co-colonization experiments have been successfully applied to determine the virulence of mutants of *Actinobacillus pleuropneumoniae* in piglets (Fuller *et al.*, 2000). Moreover, we recently successfully used this procedure to determine the virulence of an isogenic knockout of a gene encoding a fibronectin- and fibrinogen-binding protein (de Greeff *et al.*, 2002). The data clearly showed that the *lsp* mutant strain was capable of colonizing both the tonsil and the organs specific for an *S. suis* infection, as efficiently as the wild-type strain. This means that both strains are equally virulent, and that the knockout mutant of *lsp* is not attenuated *in vivo*. Thus this phenotype contrasts that of other Gram-pathogens defective in enzymes necessary for lipoprotein biosynthesis (Mei *et al.*, 1997; Coulter *et al.*, 1998; Petit *et al.*, 2001). This could suggest that lipoproteins do not play a role in the pathogenicity of *S. suis* but may also indicate that lipoproteins can be processed via an alternative route, independently of Lsp. Our data (Fig. 4) indicate that the PsaA homologue at least is not processed by an alternative path but several other lipoproteins radiolabelled with palmitic acid may be (Fig. 4). Based on Southern blot experiments, we have no reason to assume that a second Lsp gene is present in *S. suis*, and BLAST searches of the draft *S. suis* genome indicate that there is only a single copy of Lsp (i.e. the sequence reported herein). Thus the processing of lipoproteins in strain 10ΔLsp may be due to the presence of cryptic sites for other signal peptidases in some lipoprotein signal peptides. Alternatively, it may be that as yet undescribed pathway(s) for processing of lipoproteins exist in Gram-positive bacteria. This idea is supported by the observations of Tjalsma *et al.* (1999a), who showed that mature-like forms of the lipoprotein PrsA were still found in a knockout mutant of *lsp* in *B. subtilis*. Whilst these authors excluded the possibility of processing by type I signal peptidases taking over the function of Lsp, how the mature-like forms were alternatively processed

could not be explained. Thus, based on our data, and the information available in the literature, we hypothesize that in *S. suis* alternative processing of lipoproteins can also take place.

In conclusion, we describe the cloning and characterization of the prolipoprotein signal peptidase of *S. suis*, Lsp. We also show that an isogenic mutant of *lsp* is not attenuated *in vivo* in piglets. Further research is necessary to determine whether lipoproteins can be alternatively processed in *S. suis*.

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REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J. H., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Arends, J. P. & Zanen, H. C. (1988). Meningitis caused by *Streptococcus suis* in humans. *Rev Infect Dis* **10**, 131–137.

Berry, A. M. & Paton, J. C. (1996). Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*. *Infect Immun* **64**, 5255–5262.

Bolotin, A., Wincker, P., Mauger, S., Jaillon, O., Malarme, K., Weissenbach, J., Ehrlich, S. D. & Sorokin, A. (2001). The complete genome sequence of the lactic acid bacterium *Lactococcus lactis* ssp. *lactis* IL1403. *Genome Res* **11**, 731–753.

Brown, J. S., Gilliland, S. M. & Holden, D. W. (2001). A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* **40**, 572–585.

Burnette-Curley, D., Wells, V., Viscount, H., Munro, C. L., Fenno, J. C., Fives-Taylor, P. & Macrina, F. L. (1995). FimA, a major virulence factor associated with *Streptococcus sanguis* endocarditis. *Infect Immun* **63**, 4669–4674.

Clifton-Hadley, F. A. (1983). *Streptococcus suis* type 2 infections. *Br Vet J* **139**, 1–5.

Conrad, J., Sun, D., Englund, N. & Ofengand, J. (1998). The *rluC* gene of *Escherichia coli* codes for a pseudouridine synthase that is solely responsible for synthesis of pseudouridine at positions 955, 2504, and 2580 in 23S ribosomal RNA. *J Biol Chem* **273**, 18562–18566.

Coulter, S. N. Schwan W. R., Ng, E. Y. W. & 7 other authors (1998). *Staphylococcus aureus* genetic loci impacting growth and survival in multiple infection models. *Mol Microbiol* **30**, 393–404.

de Greeff, A., Buys, H., Verhaar, R., Dijkstra, J., van Alphen, L. & Smith, H. E. (2002). Contribution of fibronectin-binding protein in pathogenesis of *Streptococcus suis* serotype 2. *Infect Immun* **70**, 1319–1325.

Demple, B. (1999). Radical ideas: genetic responses to oxidative stress. *Clin Exp Pharmacol Physiol* **26**, 64–68.

Fuller, T. E., Martin, S., Teel, J. F., Alaniz, G. R., Kennedy, M. J. & Lowery, D. E. (2000). Identification of *Actinobacillus pleuropneumoniae* virulence genes using signature-tagged mutagenesis in a swine infection model. *Microb Pathog* **29**, 39–51.

Gan, K., Gupta, S. D., Sankaran, K., Schmidt, M. B. & Wu, H. C. (1993). Isolation and characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in prolipoprotein modification. *J Biol Chem* **268**, 16544–16550.

Gupta, S. D., Gan, K., Schmidt, M. B. & Wu, H. C. (1993). Characterization of a temperature-sensitive mutant of *Salmonella typhimurium* defective in apolipoprotein *N*-acyltransferase. *J Biol Chem* **268**, 16551–16556.

Hamilton, A., Harrington, D. & Sutcliffe, I. C. (2000). Characterisation of acid phosphatase activities in the equine pathogen *Streptococcus equi*. *Syst Appl Microbiol* **23**, 325–329.

Harrington, D. J., Greated, J. S., Chanter, N. & Sutcliffe, I. C. (2000). Identification of lipoprotein homologues of pneumococcal PsaA in the equine pathogens *Streptococcus equi* and *Streptococcus zooepidemicus*. *Infect Immun* **68**, 6048–6051.

Horinouchi, S. & Weisblum, B. (1982a). Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide., lincosamide. *J Bacteriol* **150**, 804–814.

Horinouchi, S. & Weisblum, B. (1982b). Nucleotide sequence and functional map of pC194. a plasmid that specifies inducible chloramphenicol resistance. *J Bacteriol* **150**, 815–825.

Inukai, M., Takeuchi, M., Shimizu, K. & Arai, M. (1978). Mechanism of action of globomycin. *J Antibiot* **31**, 1203–1205.

Kitten, T., Munro, C. L., Michalek, S. M. & Macrina, F. L. (2000). Genetic characterisation of a *Streptococcus mutans* LraI family operon and its role in virulence. *Infect Immun* **68**, 4441–4451.

Konings, R. N. H., Verhoeven, E. J. M. & Peeters, B. P. H. (1987). pKUN vectors for the separate production of both DNA strands of recombinant plasmids. *Methods Enzymol* **153**, 12–34.

Koskiniemi, S., Sellin, M. & Norgren, M. (1998). Identification of two genes, *cpsX* and *cpsY*, with putative regulatory function on capsule expression in group B streptococci. *FEMS Immunol Med Microbiol* **21**, 159–168.

Mahan, M. J., Slauch, J. M. & Mekalanos, J. J. (1993). Selection of bacterial virulence genes that are specifically induced in host tissues. *Science* **259**, 686–688.

Marra, A., Lawson, S., Asundi, J. S., Brigham, D. & Hromockyj, A. E. (2002). *In vivo* characterisation of the *psa* genes from *Streptococcus pneumoniae* in multiple models of infection. *Microbiology* **148**, 1483–1491.

Mei, J.-M., Nourbakhsh, F., Ford, C. W. & Holden, D. W. (1997). Identification of *Staphylococcus aureus* virulence genes in a murine model of bacteraemia using signature-tagged mutagenesis. *Mol Microbiol* **26**, 399–407.

Miller, J. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Petit, C. M., Brown, J. R., Ingraham, K., Bryant, A. P. & Holmes, D. J. (2001). Lipid modification of prelipoproteins is dispensable for growth in vitro but essential for virulence in *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **200**, 229–233.

Prágai, Z., Tjalsma, H., Bolhuis, A., van Dijl, J. M., Venema, G. & Bron, S. (1997). The signal peptidase II (*lsp*) gene of *Bacillus subtilis*. *Microbiology* **143**, 1327–1333.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sankaran, K. & Wu, H. C. (1994a). Lipid modification of bacterial prolipoprotein – transfer of diacylglycerol moiety from phosphatidylglycerol. *J Biol Chem* **269**, 19701–19706.

Sankaran, K. & Wu, H. C. (1994b). Signal peptidase II – specific signal peptidase for bacterial lipoproteins. In *Signal Peptidases*, pp. 17–29. Edited by G. von Heijne. Austin: R. G. Landes.

Smith, H. E., Vecht, U., Gielkens, A. L. J. & Smits, M. A. (1992). Cloning and nucleotide sequence of the gene encoding the

136-kilodalton surface protein (muramidase-released protein) of *Streptococcus suis* type 2. *Infect Immun* **60**, 2361–2367.

Smith, H. E., Wisselink, H. J., Vecht, U., Gielkens, A. L. J. & Smits, M. A. (1995). High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. *Microbiology* **141**, 181–188.

Smith, H. E., Damman, M., van der Velde, J., Wagenaar, F., Wisselink, H. J., Stockhofe-Zurwieden, N. & Smits, M. A. (1999a). Identification and characterization of the *cps* locus of *Streptococcus suis* serotype 2: the capsule protects against phagocytosis and is an important virulence factor. *Infect Immun* **67**, 1750–1756.

Smith, H. E., Veenbergen, V., van der Velde, J., Damman, M., Wisselink, H. J. & Smits, M. A. (1999b). The *cps* genes of *Streptococcus suis* serotypes 1, 2 and 9: development of rapid serotype-specific PCR assays. *J Clin Microbiol* **37**, 3146–3152.

Smith, H. E., Buijs, H., de Vries, R., Wisselink, H. J., Stockhofe-Zurwieden, N. & Smits, M. A. (2001). Environmentally regulated genes of *Streptococcus suis*: identification by the use of iron-restricted conditions *in vitro* and by experimental infection of piglets. *Microbiology* **147**, 271–280.

Sutcliffe, I. C. & Harrington, D. J. (2002). Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology* **148**, 2065–2077.

Sutcliffe, I. C. & Russell, R. R. B. (1995). Lipoproteins of gram-positive bacteria. *J Bacteriol* **177**, 1123–1128.

Tettelin, H., Nelson, K. E., Paulsen, I. T. & 36 other authors (2001). Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* **293**, 498–506.

Tjalsma, H., Kontinen, V. P., Prágai, Z., Wu, H., Meima, T., Venema, G., Bron, S., Sarvas, M. & van Dijl, J. M. (1999a). The role of lipoprotein processing by signal peptidase II in the Gram-positive eubacterium *Bacillus subtilis*. *J Biol Chem* **274**, 1698–1707.

Tjalsma, H., Zanen, G., Venema, G., Bron, S. & van Dijl, J. M. (1999b). The potential active site of the lipoprotein-specific (type II) signal peptidase of *Bacillus subtilis*. *J Biol Chem* **274**, 28191–28197.

Tokunaga, M., Loranger, J. M. & Wu, H. C. (1983). Isolation and characterization of an *Escherichia coli* clone overproducing prolipoprotein signal peptidase. *J Biol Chem* **258**, 12102–12105.

Vecht, U., van Leengoed, L. A. M. G. & Verheyen, E. R. M. (1985). *Streptococcus suis* infections in pigs in The Netherlands (part I). *Vet Q* **7**, 315–321.

Vecht, U., Arends, J. P., van der Molen, E. J. & van Leengoed, L. A. M. G. (1989). Differences in virulence between two strains of *Streptococcus suis* type 2 after experimentally induced infection of newborn germfree pigs. *Am J Vet Res* **50**, 1037–1043.

Vecht, U., Wisselink, H. J., van Dijk, J. E. & Smith, H. E. (1992). Virulence of *Streptococcus suis* type 2 strains in newborn germfree pigs depend on phenotype. *Infect Immun* **60**, 550–556.

Williams, M. G., Fortson, M., Dykstra, C. C., Jensen, P. & Kushner, S. R. (1989). Identification and genetic mapping of the structural gene for an essential *Escherichia coli* membrane protein. *J Bacteriol* **171**, 565–568.

Wisselink, H. J., Reek, F. H., Vecht, U., Stockhofe-Zurwieden, N., Smits, M. A. & Smith, H. E. (1999). Detection of virulent strains of *Streptococcus suis* type 2 and highly virulent strains of *Streptococcus suis* type 1 in tonsillar specimens of pigs by PCR. *Vet Microbiol* **67**, 143–157.

Wisselink, H. J., Joosten, J. J. & Smith, H. E. (2002). Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens of pigs. *J Clin Microbiol* **40**, 2922–2929.

Witke, C. & Götz, F. (1995). Cloning and nucleotide sequence of the signal peptidase II (*lsp*)-gene from *Staphylococcus carnosus*. *FEMS Microbiol Lett* **126**, 233–240.

Yamagata, H., Ippolito, C., Inukai, M. & Inouye, M. (1982). Temperature-sensitive processing of outer membrane lipoprotein in an *Escherichia coli* mutant. *J Baceteriol* **152**, 1163–1168.

Zhao, X.-J. & Wu, H. C. (1992). Nucleotide sequence of the *Staphylococcus aureus* signal peptidase II (*lsp*) gene. *FEBS Lett* **299**, 80–84.