Identification of virulence factors of Streptococcus suis

de Greeff, A.

Publication date
2002

Chapter 6

Lipoprotein Signal Peptidase of *Streptococcus suis* serotype 2

Astrid de Greeff, Herma Buys, Loek van Alphen, and Hilde E. Smith

Accepted after revision in Microbiology UK.

Abstract

In this study, we describe the complete coding sequence for a prolipoprotein signal peptidase (SP-ase II) of *S. suis*, LspS. This is 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, we showed that the *lspS*-gene was transcribed *in vitro*. Functionality of LspS in *E. coli* was demonstrated by using an *in vitro* globomycin resistance assay, to show that expression of LspS in *E. coli* increased the globomycin resistance. An isogenic mutant of *S. suis* serotype 2 unable to produce LspS was constructed. Five piglets were inoculated with a mixture of both strains in an experimental infection, to determine the relative virulence of the mutant strain compared to that of the wild-type strain in a competitive challenge experiment. The data showed that both strains were equally virulent, indicating that the knock-out mutant of *lspS* is not attenuated *in vivo*.

Introduction

Streptococcus suis is a gram-positive organism that is an important cause of meningitis, arthritis, and septicemia 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 that are sensitive for the infection. Occasionally, humans get infected by *S. suis* (Arends and Zanen, 1988). Control of the disease is hampered by the lack of effective vaccines, and sufficient, sensitive diagnostic tools. Recently, PCR assays were developed for the detection of serotypes 1, 2, 7, and 9 (Wisselink *et al.*, 1999; Smith *et al.*, 1999). For other serotypes no sensitive diagnostics are available yet. For development of vaccines and diagnostics, more insight into the process of pathogenesis is 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*, *cpsY* is not linked to the capsule operon (Smith *et al.*, 1999), we here determined if in *S. suis* this regulator belongs to an operon, and the genes flanking *cpsY* in *S. suis*.

In this paper we describe the operon expressing the transcriptional regulator and show that the operon contains a prolipoprotein signal peptidase. This signal peptidase type II of *S. suis* (*lspS*), and the operon it is part of, were cloned and characterized. Functionality of LspS was demonstrated in *E. coli*. An insertional knock-out mutant of *lspS* was constructed and the role of the prolipoprotein signal peptidase in the pathogenesis of *S. suis* was studied by comparing wild-type and mutant strain in an experimental infection in piglets. The data show that the *lspS* mutant is not attenuated *in vivo*.

Materials and 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, London, United

Kingdom) 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: 3.4 μg ml^{-1} of chloramphenicol (Roche, Mannheim, Germany) for *S. suis*, and 10 μg ml^{-1} for *E. coli*, and 100 μg ml^{-1} of ampicillin (Roche) for *E. coli*.

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Relevant characteristics	Source/reference
Strains		
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' proAB *lacI*^{q}ZΔM15 Tn10 (Tet^{R})]	Stratagene
S. suis		
10	Virulent serotype 2 strain	Vecht *et al.*, 1989
10ΔLspS	Isogenic *lspS* mutant of strain 10	This work
Plasmids		
pGEM7Zf(+)	Replication functions pUC, Amp^{R}	Promega Corp.
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 and Weisblum, 1982a
pUK21	Replication functions 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 and Weisblum, 1982b
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
pLSPS-2	pGEM7Zf(+) containing 3 kb *DraI-ClaI* fragment of *lspS*	This work
pLSPS-3	pLSPS-2 in which a Cm -resistance cassette preceded by the MRP-promoter was inserted into the unique *StuI* site	This work

Tet^{R} tetracyclin resistance; Amp^{R} ampicillin resistance; Cm^{R} chloramphenicol resistance; Em^{R} erythromycin resistance

***In vitro* transcription/translation assay.** *In vitro* transcription/translation assay was performed using the Prokaryotic DNA-Directed Translation Kit (Amersham Life Science, Buckinghamshire, Great Britain) according to manufacturer's instructions. TRAN^{35}S-LABEL™ (containing 70% L-methionine, [^{35}S]) (43.5

GBq/mmol; ICN Biomedicals, Irvine, Ca., USA) was used to label *de novo* synthesized proteins. After the *in vitro* transcription/translation, SDS-PAGE was performed with the NuPAGE® system according to instructions of the manufacturer. 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 phosphorimager (STORM, Molecular Dynamics, Sunnyvale, Calif.).

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. 10 µl 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 optical density at 630 nm 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, Warrington, Great Britain). Samples were prepared by use of an ABI Prism dye terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequencing data were assembled and analyzed using the Lasergene program (DNASTAR). The BLAST software package was used to search for protein sequences homologous to the deduced amino acid sequences in the GenBank/EMBL databases.

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, Boston, USA) as described by Sambrook *et al.* (1989). DNA probes of the *lspS* and chloramphenicol resistance genes were labeled with [α-^{32}P]dCTP (111 GBq/mmol; Amersham Life Science, Buckinghamshire, Great Britain) by use of a random primed DNA labeling 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% sodium dodecyl sulphate. After hybridization, the membranes were washed twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 5% sodium dodecyl sulphate at 65°C for 30 min and twice with a buffer containing 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 1% sodium dodecyl sulphate at 65°C for 30 min. The signal was detected using a phosphor-imager (Storm, Molecular Dynamics, Sunnyvale, Calif.).

Construction of an *lspS* knock-out mutant. To construct the mutant strain 10ΔLspS, the pathogenic strain 10 (Vecht *et al.*, 1989; Vecht *et al.*, 1992) of *S. suis* serotype 2 was electrotransformed (Smith *et al.*, 1995) with the plasmid pLSPS-3 (Figure 1). In this plasmid the *lspS* gene was inactivated by the insertion of a resistance cassette, consisting of the chloramphenicol resistance gene from pC194 (Horinouchi & Weisblum, 1982a), preceded by the promoter-region of the *mrp* gene (Smith *et al.*, 1992). To create pLSPS-3, pLSPS2 (Figure 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 transformated 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 ml^{-1} of ampicillin and 10 µg ml^{-1} of chloramphenicol. After electrotransformation of strain 10 with pLSPS-3, chloramphenicol resistant colonies were selected on Columbia blood base agar plates containing 3.4 µg ml^{-1} of chloramphenicol. Southern blotting and hybridization experiments were used to select for double crossover integration events (data not shown).

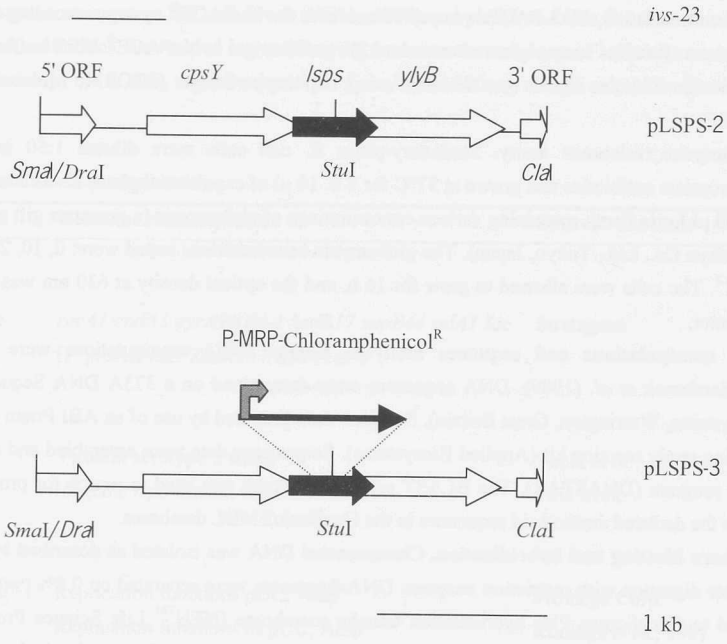

Figure 1. Schematic representation of pLSPS-2 and pLSPS-3. A 3 kb *Cla*I-*Dra*I fragment that hybridized with probe *ivs-23/iri-24*, was cloned into pGEM7Zf(+) digested with *Cla*I and *Sma*I, yielding pLSPS-2. To create pLSPS-3, the 900 bp *Sma*I-*Eco*RV fragment of pUK21-Cm, containing the chloramphenicol resistance gene from pC194 (Horinouchi and Weisblum, 1982), preceded by the promoter region of the *mrp* gene (Smith *et al.*, 1992) was inserted in the *Stu*I site of *lspS*.

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; Vecht *et al.*, 1992). Six-day-old piglets were inoculated intranasally with about 10^7 cfu of *Bordetella bronchiseptica* 92932, to predispose the piglets to infection with *S. suis*. Two days later the pigs were inoculated intranasally with 10^6 CFU of *S. suis* strain 10 plus 10^6 CFU of *S. suis* strain 10ΔLspS. 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. For ethical reasons this 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 feces were cultured three times a week. The swabs were plated directly onto Columbia agar containing 6% horse blood, or grown for 48 hr in Todd-Hewitt broth and subsequently again plated onto Columbia agar containing 6% horse blood. Pigs were monitored twice a day for clinical signs and symptoms, such as fever,

nervous signs, and lameness. Blood samples from each pig 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 the pigs 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 (CNS), serosae, and joints) were sliced with a scalpel or a tissuemizer. Tissue slices from each organ or site were resuspended in 2 - 25 ml of Todd-Hewitt containing 15% glycerol, depending on the size of the tissue slice. The suspension was centrifuged at 1,200 x *g* for 5 min. The supernatant was collected and serial dilutions were plated on Columbia agar plates containing 6% horse blood, as well as on Columbia agar plates containing 6% horse blood and 3.4 μg/ml of chloramphenicol to quantitate the number of wild-type and mutant bacteria present. The number of mutant strain 10ΔLspS cells was determined by counting the number of CFU on the appropriate serial dilution on the selective plates; the number of wild-type strain 10 cells was determined by counting the number of CFU on the appropriate serial dilution on the Columbia Agar blood plates of which the number of CFU 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 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 ml^{-1} of chloramphenicol.

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.

Nucleotide sequence accession number. The nucleotide sequence data of *lsps* have been submitted to GenBank under accession no. AF303229.

Results

***Lsps* is located in an operon.** One of the clones selected from a genomic library of *S. suis* serotype 2, was both induced *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 *Escherichia coli*. A ^{32}P labeled 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 pLSPS-2 (Figure 1). The complete sequence of the insert was determined. Examination of the sequence revealed the presence of 3 complete and partially overlapping open reading frames (ORFs), and 2 incomplete ORFs (Figure 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 RBS' 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 operon encoded a 304 aa protein that showed similarity to the regulators CpsY/OxyR described above. The second ORF of the operon encoded a 154 aa protein, that showed similarity to a putative prolipoprotein signal peptidase of *S. pyogenes*, and the last ORF of the operon encoded a 297 aa protein, that was similar to YlyB of *Bacillus subtilis*.

Analysis of LspS of *S. suis*. The protein sequence of LspS was compared to other signal peptidase II sequences present in the database. LspS was most similar to SP-ase II of *S. pyogenes* (48% identity) (acc. no. AE006533), *L. lactis* (45%) (acc. no. AAK050950), *S. carnosus* (39%) (Witke & Götz, 1995), and *B. subtilis* (35%) (Prágai *et al.*, 1997). A hydrophobicity plot (Kyte-Doolittle) revealed the presence of four hydrophobic regions, suggesting a similar membrane localization for LspS as described for other SP-ases II (Witke & Götz, 1995; Prágai *et al.*, 1997). In SP-ase II the aspartic acid residue on position 17 is necessary for signal peptidase II activity in *E. coli* (Sankaran & Wu, 1995). This residue is conserved among the SP-ase II of many organisms, including *S. suis*. Most signal peptidases have a cluster of positively charged residues at their C-terminus (Innis *et al.*, 1984). In LspS one lysin residue was found.

LspS is expressed *in vitro*. Expression of LspS was determined by using an *E. coli* based *in vitro* transcription/translation system on pLSPS-2. pGEM7Zf(+) was used as a negative control. Figure 2 shows that 3 proteins were expressed corresponding very well to the predicted sizes of CpsY, YlyB and LspS of respectively 34.7 kDa, 32.8 kDa, and 17.5 kDa. The negative control pGEM7Zf(+) expressed the α-lacZ fragment as well as β-lactamase (amp). These data clearly showed that all 3 genes were expressed very efficiently from pLSPS-2 (Figure 2).

Globomycin resistance. To test whether LspS was functional in *E. coli*, we used a globomycin assay. Globomycin is a cyclic peptide antibiotic that specifically inhibits the processing of prolipoprotein 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.

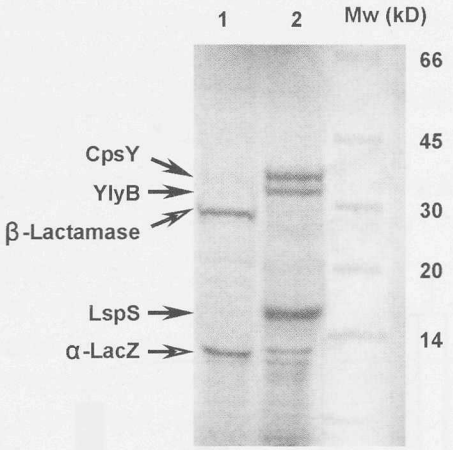

Figure 2. *In vitro* transcription/ translation assay. Lane 1: pGEM7Zf(+), negative control; Lane 2: pLSPS-2; Mw (kD): ^{14}C methylated rainbow marker (Amersham Life Science, Buckinghamshire, UK), sizes in kiloDalton. Open arrows indicate proteins expressed from the control plasmid pGEM7Zf(+). Closed arrows indicate proteins expressed by pLSPS-2.

pLSPS-2 was used for the expression of LspS. In pLSPS-3, a chloramphenicol resistance cassette was inserted into the *lspS* gene to inactivate the gene (Figure 1). 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 optical density of the cultures was measured. Figure 3 shows that *E. coli* harboring pGEM7Zf(+), or pLSPS-3 did not grow in the presence of a concentration of globomycin higher than 20 - 40 μg ml^{-1}. In contrast, *E. coli* harboring pLSPS-2, could grow in the presence of a concentration of globomycin higher than 320 μg ml^{-1} (Figure 3), although the maximum optical density measured for this strain is lower than for the other strains. This indicates that the expression of LspS and/or YlyB slightly inhibited the growth of *E. coli*. The increase of globomycin resistance due to expression of LspS demonstrates that *lspS* indeed encodes an SP-ase II that is functional in *E. coli*.

Role of LspS in pathogenesis of *S. suis* serotype 2. To test the role of LspS in the pathogenesis of *S. suis*, an isogenic knock-out mutant of LspS was constructed in strain 10, yielding strain 10ΔLspS. Strain 10ΔLspS was constructed by electroporation of pLSPS-3, in which the *lsps* gene was inactivated by insertion of a chloramphenicol resistance cassette

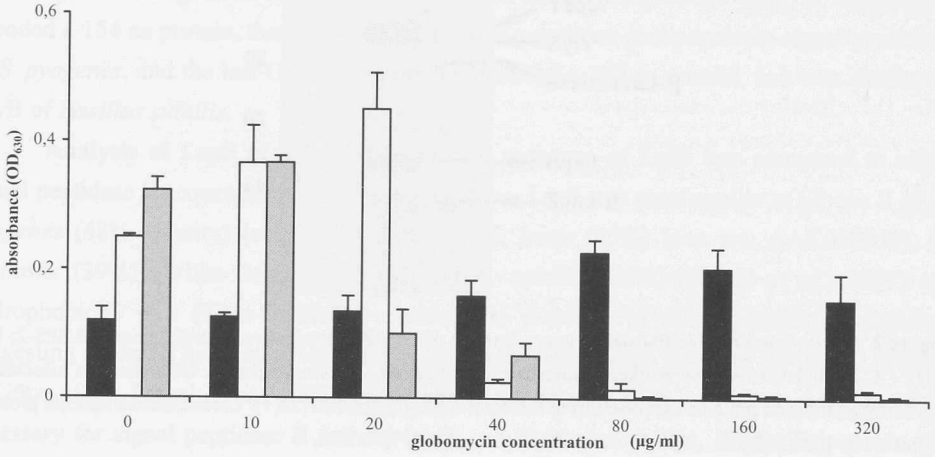

Figure 3. Globomycin resistance assay. Optical Density (OD_{630}) of *E. coli* cultures harboring different plasmids was measured after 16 hr of growth. Black bars: pLSPS-2; white bars: pGEM7Zf(+); gray bars: pLSPS-3. Bars represent the mean of triplicate experiments, error bars indicate the standard error of the mean (SEM).

(Figure 1), into *S. suis* (Smith *et al.*, 1995). Southern blotting and hybridization experiments were used to select for double crossover integration events (data not shown). 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 10ΔLspS was compared to that of the wild-type in an experimental infection in piglets. The mutant strain 10ΔLspS 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 CFU of strain 10 and 4×10^5 CFU of strain 10ΔLspS), in five piglets (no. 1, 26, 42, 44, and 49). During the experiment, piglets that developed specific *S. suis* symptoms (meningitis, arthritis, or mortally 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 (no. 26, 42 and 44), one of them also developed a severe pericarditis (no. 44). Piglet no. 49 developed a severe arthritis

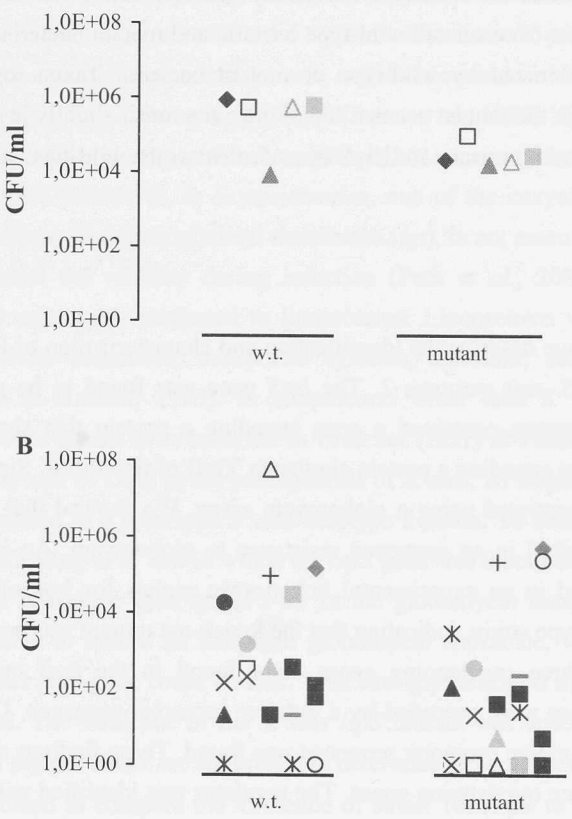

Figure 5. Efficacy of colonization of wild-type and mutant bacteria on various organs of infected pigs. Panel A depicts colonization of wild-type strain 10 and mutant strain 10ΔLspS on the tonsils. ◆ tonsil piglet no. 1; □ tonsil piglet no 26; ▲ tonsil piglet no 42; △ tonsil piglet no 44; ■ tonsil piglet no 49. Panel B depicts colonization of the specific organs. ▲ CNS piglet no 1; ■ joints piglet no 1; ● peritonitis 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; ○ pericard piglet no 44; ● spleen piglet no 49; ▲ heart piglet no 49; ■ joint piglet no 49. Each dot represents the numbers of wild-type or mutant bacteria isolated from one particular organ, from one pig.

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 figure 5A, wild-type and mutant bacteria were reisolated 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 that the wild-type bacteria. Figure 5B shows that no large differences were found in the ratio of reisolated wild-type bacteria and mutant bacteria, nor in 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 10ΔLspS is as virulent as the wild-type strain.

Discussion

In this paper we describe the identification and characterization of LspS, a lipoprotein signal peptidase of *S. suis* serotype 2. The *lspS* gene was found to be part of an operon. Besides *lspS*, this operon contained a gene encoding a protein that showed similarity to regulators, and a gene encoding a protein similar to YlyB of *B. subtilis*. Signal peptidase type II activity was demonstrated using a globomycin assay. We showed that overexpression of LspS in *E. coli* resulted in an increased resistance to globomycin. An isogenic knock-out mutant of *lspS* tested in an experimental infection in piglets for loss of virulence was as virulent as the wild-type strain, indicating that the knock-out mutant was not attenuated.

In *S. suis*, three overlapping genes were found in the *lspS* operon, including a regulator. These genes were preceded by a putative promoter sequence. Downstream of the third gene another putative promoter sequence was found. These findings suggest this operon contains only the three overlapping genes. The 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 this operon, containing the regulator, in the pathogenesis of *S. suis* serotype 2. The role of the regulator found upstream of *lspS* is unknown. The second gene was similar to a lipoprotein signal peptidase. Like in *B. subtilis*, *lspS* in *S. suis* is followed by *ylyB*, and similar to the situation in *B. subtilis*, these genes overlap. No role was attributed to *ylyB*. In contrast to other organisms, *lspS* in *S. suis* is preceded by a regulator. In *B. subtilis*, *lsp* is preceded by *ylyA* and *ileS*, respectively (Tjalsma *et al.*, 1999). In other organisms, *lsp* is often preceded by *ileS*.

Prolipoprotein signal peptidase, also called signal peptidase II, is involved in the removal of the signal peptide from glyceride-modified prolipoproteins (Sankaran & Wu, 1994a+b). Subsequently, an additional acyl group is attached to the NH_2-terminal cysteine by

N-acyl transferase and the lipoprotein is anchored to the cell wall. In gram-negative organisms several enzymes involved in the lipid-modification of prolipoproteins, among which SP-ase II, 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). On the contrary, *lsp* of the gram-positive *B. subtilis* is not necessary for growth and viability (Tjalsma *et al.*, 1999). Moreover, in *S. pneumoniae*, one of the enzymes involved in lipid-modification, prolipoprotein diacylglyceryl transferase (*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 were attributed to lipoproteins. Lipoproteins were described to be involved in antibiotic resistance, transporter systems, adhesion, secretion, and sensory systems (Sutcliffe & Russell, 1995). In streptococci other than *S. suis*, several of the lipoproteins have been shown to be involved in virulence (Berry & Paton, 1996; Brown *et al.*, 2001). To study the role of LspS in the pathogenesis of *S. suis*, an isogenic knock-out mutant of *lspS* 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 *lspS* gene was inactivated by the insertion of a chloramphenicol resistance gene (pLSPS-3). In the globomycin assay, *E. coli* containing pLSPS-3, was unable to induce an increased globomycin resistance, whereas the construct containing the intact *lspS* gene could do this. This strongly indicated that in pLSPS-3, LspS was non-functional. The virulence of the *S. suis lspS* 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 10ΔLspS to the wild-type *S. suis* strain 10 in a competitive co-colonization assay in piglets. This kind 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 knock-out of a gene encoding a fibronectin- and fibrinogen binding protein (de Greeff *et al.*, 2002). The data clearly showed that the mutant strain 10ΔLspS 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 knock-out mutant of *lspS* is not attenuated *in vivo*. Two possible explanations can be envisaged for this observation. Firstly, SP-ase II, and thus lipoproteins do not play a role in the pathogenesis, or secondly lipoproteins can be processed via an alternative route, and as a result the lipoproteins are still expressed on the surface. Since lipoproteins of other streptococci play a role in the pathogenesis of streptococcal disease (Petit *et al.*, 2001), the first option seems not very likely. PsaA is a

lipoprotein of *S. pneumoniae*, that was described to be involved in adhesion and that is essential for virulence (Berry and Paton, 1996). An ABC-transporter of *S. pneumoniae* was described, of which the receptor part was a lipoprotein, that was shown to be involved in virulence (Brown *et al.*, 2001).

The second possibility is, that lipoproteins can be processed via an alternative routing, independently of LSPS. This idea is supported by the observation of Tjalsma *et al.*, 1999. These authors showed that, in a knock-out mutant of *lsp* in *B. subtilis*, still mature-like forms of the lipoprotein PrsA were found (Tjalsma *et al.*, 1999). The authors excluded the possibility of SP-ase I taking over the function of SP-ase II, but could not explain how the proteins were alternatively processed. Until now, only one category of SP-ase II genes was described in literature. Based on Southern blot experiments, we have no reason to assume that a second SP-ase II gene is present in *S. suis*, although we cannot exclude the possibility of a second SP-ase II gene with a completely different sequence/structure. Based on our data, and the information available in the literature, we hypothesize that in *S. suis* also alternative processing of lipoproteins takes place.

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

Acknowledgements

We thank the people from the Animal and Laboratory Services and the people from Pathobiology of the ID-Lelystad for their assistance during the animal experiments.

References

Arends, J. P., and H. C. Zanen 1988. Meningitis caused by *Streptococcus suis* in humans. Rev. Infect. Dis. **10:**131-137.

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

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

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

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

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

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

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

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

Horinouchi, S., and B. Weisblum. 1982a. Nucleotide sequence and functional map of pC194, a plasmid that specifies inducible chloramphenicol resistance. J. Bacteriol. **150:**815-825.

Horinouchi, S., and B. Weisblum. 1982b. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibodies. J. Bacteriol. **150:**804-814.

Innis, M. A., M. Tokunaga, M. E. Williams, J. M. Loranger, S. Y. Chang, S. Chang, and H. C. Wu. 1984. Nucleotide sequence of the *Escherichia coli* prolipoprotein signal peptidase (lsp) gene. Proc. Natl. Acad. Sci. USA **81:**3708- 3712.

Inukai, M., M. Takeuchi, K. Shimizu, and M. Arai. 1978. Mechanism of action of globomycin. J. Antibiot. **31:**1203-1205.

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

Koskiniemi, S., M. Sellin, and M. Norgren. 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., J. M. Slauch, and J. J. Mekalanos. 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686-688.

Miller, J. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory. Cold Spring Harbor. N.Y.

Petit, C. M., J. R. Brown, K. Ingraham, A. P. Bryant, and D. J. Holmes. 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., H. Tjalsma, A. Bolhuis, J. M. van Dijl, G. Venema, and S. Bron. 1997. The signal peptidase II (*lsp*) gene of *Bacillus subtilis*. Microbiology **143:**1327-1333.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor. N.Y.

Sankaran, K., and H. C. Wu. 1994a. Lipid modification of bacterial prolipoprotein - transfer of diacylglycerol moiety from phosphatidylglycerol. J. Biol. Chem. **269:**19701-19706.

Sankaran, K., and H. C. Wu. 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 Company.

Sankaran, K., and Wu. 1995. Bacterial prolipoprotein signal peptidase. Meth. Enzym. **248:**169-180.

Smith, H. E., H. Buijs, R. de Vries, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits. 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.

Smith, H. E., M. Damman, J. van der Velde, F. Wagenaar, H. J. Wisselink, N. Stockhofe-Zurwieden, and M. A. Smits. 1999. 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., U. Vecht, A. L. J. Gielkens, and M. A. Smits. 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., H. J. Wisselink, U. Vecht, A. L. J. Gielkens, and M. A. Smits. 1995. High-efficiency transformation and gene inactivation in *Streptococcus suis* type 2. Microbiology **141:**181-188.

Smith, H. E., V. Veenbergen, J. van der Velde, M. Damman, H. J. Wisselink, and M. A. Smits. 1999. The *cps* genes of *Streptococcus suis* serotypes 1, 2 and 9: development of rapid serotype-specific PCR assays. J. Clin. Microbiol. **37:**3146-52.

Sutcliffe, I. C., and R. R. B. Russell. 1995. Lipoproteins of gram-positive bacteria. J. Bacteriol. **177:**1123-1128.

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

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

Vecht, U., J. P. Arends, E. J. van der Molen, and L. A. M. G. van Leengoed. 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., L. A. M G. van Leengoed, and E. R. M. Verheyen. 1985. *Streptococcus suis* infections in pigs in The Netherlands (part I). Vet. Q. **7:**315-321.

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

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

Wisselink, H. J., F. H. Reek, U. Vecht, N. Stockhofe-Zurwieden, M. A. Smits, and H. E. Smith. 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.

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

Yamagata, H., C. Ippolito, M. Inukai, and M. Inouye. 1982. Temperature-sensitive processing of outer membrane lipoprotein in an *Escherichia coli* mutant. J. Bacteriol. **152:**1163-1168.

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