Characterization of LppS, an adhesin of Mycoplasma conjunctivae

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A serine-rich membrane protein named LppS from Mycoplasma conjunctivae, the aetiologic agent of infectious keratoconjunctivitis (IKC) of domestic and wild Caprinae, was characterized. Gene cloning and sequence analysis of the lppS gene revealed that it encoded a membrane protein precursor. The protein had a typical signal sequence and a signal peptidase II cleavage site followed by a cysteine residue representing a potential acylation site. The mature LppS protein had an apparent molecular mass of 150 kDa and was found in the detergent-associated fraction of Tween 20 extracted M. conjunctivae proteins. It possessed a serine-rich domain of 41 aa with 37 (90·2 %) serine residues. Twenty-seven of these serine residues were contiguous. The protein adhered to lamb joint synovial cells. Using an in vitro adhesion model, Fab fragments from IgG directed against recombinant purified LppS were shown to specifically inhibit adhesion of M. conjunctivae to lamb cells. Thus, LppS is likely to be an adhesin of M. conjunctivae that may play an important role in the pathogenesis of IKC.

INTRODUCTION

Mycoplasma conjunctivae is known to be the primary cause of infectious keratoconjunctivitis (IKC) in Switzerland. This disease affects wild Caprinae (Mayer et al., 1997; Giacometti et al., 1998) such as chamois and ibex, and domestic sheep and goats. The disease is endemic in the Swiss sheep population (Janovsky et al., 1998) but not in wild Caprinae (Giacometti et al., 2002). Currently, IKC is monitored either by detection of M. conjunctivae in lacrimal fluids using nested PCR (Giacometti et al., 1999), or serologically by Western blot analysis (Degiorgis et al., 2000) or indirect ELISA (Belloy et al., 2001). However, there are no efficient therapeutic or preventative measures available. This is mainly due to the lack of understanding of the steps in the pathogenesis of M. conjunctivae infections such as adhesion, colonization, induction of inflammation and destruction of the host cells.

Mycoplasma cells are only delimited by a single trilaminar membrane consisting of lipids and proteins with a small amount of polysaccharide. As mycoplasmas lack a cell wall and outer membrane, their single membrane must contain the necessary factors for adherence to, and colonization of, host tissues, as well as structures capable of preventing damage by the humoral immune response. Several recent reports show the prevalence of genes capable of encoding antigenically variable surface proteins in several mycoplasma species (Rosengarten & Wise, 1990; Yogev et al., 1991; Bhugra et al., 1995; Simmons et al., 1996; Lysnyansky et al., 1996; Citti & Rosengarten, 1997). Mycoplasma surface proteins are often amphiphilic integral membrane proteins, many of which are covalently modified by lipid (Jan et al., 1996). Among them, adhesin proteins play an important role in pathogenicity (Hu et al., 1982; Krause, 1998; Boguslavsky et al., 2000; Seto et al., 2001).

Recent studies detected several specific antigens of M. conjunctivae, including proteins of 175, 73, 68, 60 and 33 kDa, which have been exploited to develop specific serological tests to detect M. conjunctivae infection (Degiorgis et al., 2000; Belloy et al., 2001). To study the antigenic proteins in more detail, we screened a bacteriophage λ-based expression gene library of the M. conjunctivae type strain HRC/581T using rabbit serum raised against a whole cell preparation of M. conjunctivae. The clones were then analysed further by DNA sequence analysis and a clone containing a gene for a potential adhesin was selected and characterized in detail.

METHODS

Strains and culture conditions. M. conjunctivae type strain HRC/581T was grown on standard mycoplasma broth medium enriched with 20 % horse serum, 2·5 % yeast extract and 1 % glucose (Axcell Biotechnologies). The cells were harvested by centrifugation at 13,000 g for 20 min, washed three times in TES buffer...
For gene cloning, *Escherichia coli* strains XL-1 Blue MRF° and XLOLR (Stratagene) were used. These strains were grown on Luria–Bertani broth at 37 °C in an orbital shaker-incubator (Sambrook et al., 1989). Antibiotics (ampicillin, 100 μg ml⁻¹; kanamycin, 50 μg ml⁻¹) were added when needed to enable selection for *E. coli* carrying recombinant plasmids.

**DNA extraction and DNA manipulation.** DNA from *M. conjunctivae* type strain HRC/581T was extracted by the guanidium thiocyanate method (Pitche et al., 1989). Ligation, subcloning, plasmid extraction of the DNA fragments, agarose gel electrophoresis (0-7 %) and photography were performed as described previously (Ausubel et al., 1999). Plasmid extraction from recombinant *E. coli* clones was done by alkaline lysis using Miniprep kits (Qiagen).

**Construction of genomic library, cloning and DNA sequence analysis.** Genomic DNA of *M. conjunctivae* HRC/581T was partially digested with *Sau*3A1 and fragments from 2 to 10 kb were selected to construct a genomic library, using *Bam*HI-digested λ-ZAP-express vector arms, which was packaged with the Gigapack-11 packaging system (Stratagene). The library was plated using standard protocols on the *E. coli* strain XL-1 Blue MRF°. Immunoscreening was carried out by blotting phage plaques onto nitrocellulose membranes and reacting them with rabbit hyperimmune serum directed against whole cell antigens of *M. conjunctivae* HRC/581T (Belloy et al., 2001). Positive clones were purified and subjected to in vivo excision using the fl helper phage in *E. coli* strain XLOLR. Both ends of the fragments inserted into the excised phagemid vector pBK-CMV were sequenced using an ampli-Taq FS dye terminator kit (Applied Biosystems) and the universal primers complementary to the T3 and T7 promoters flanking the multiple cloning site of pBK-CMV. The complete sequence of the inserted and the plasmid was obtained by deriving a double-stranded nested-deletion series using exonuclease III (Pharmacia Biotech), from approximately 500 ng plasmid DNA following the manufacturer’s protocol. DNA analysis of segments adjacent to the cloned fragment was performed using the Vectorette II System (Genosys Biotechnologies). This system is based on a unidirectional approach and the previously determined DNA sequences from *M. conjunctivae* allowed the design of specific primers. Briefly, 100 ng genomic DNA from *M. conjunctivae* was digested with different enzymes (*Bcl* I, *Hae*III, *Hind*III, *Pvu*I or *Rsa*I) and ligated to the corresponding Vectorette units as described in the protocol. Thereafter, unidirectional PCR amplification using one primer specific for the Vectorette unit and one primer complementary to the known mycoplasmal sequence (Table 1) produced a fragment which was then entirely sequenced by primer walking. Sequences were determined with an ABI Prism model 3100 genetic analyser (Applied Biosystems). DNA sequences were assembled and edited with the program Sequencher 3.0 (GeneCode).

A digoxigenin-labelled probe for the *lppS* gene was produced by PCR with the primers Ser_start2 and Ser_end (Table 1) and inclusion of 50 μM digoxigenin-11-dUTP (Roche Diagnostıc) in the reaction. Southern blot analysis was performed using standard methods (Ausubel et al., 1999) with the digoxigenin-labelled *lppS* probe.

**Colony-blot analysis.** Nitrocellulose membranes (0-45 μm; Millipore) were applied to plates with *λ* phage-infected *E. coli* MRF° strains for 15 min and blocked with 1 % milk buffer (100 mM Tris/HCl, pH 7-5, 150 mM NaCl, 0-5 % Tween 20, 1 %, w/v, skimmed milk powder) for 1 h at room temperature. Then, membranes were incubated in rabbit hyperimmune serum against total antigens from *M. conjunctivae* HRC/581T diluted 1:2000 in 1 % milk buffer, for 90 min at room temperature. The membranes were then washed with 1 % milk buffer and incubated with monoclonal goat anti-rabbit phosphatase-labelled antibodies (Kirkegaard & Perry Laboratories) at a dilution of 1:2000 in 1 % milk buffer for 90 min at room temperature with shaking. The bound conjugate was detected by incubation in nitro blue tetrazolium and bromochloroindolyl phosphate (NBT/BCIP) in alkaline substrate buffer (7 mM Na₂CO₃, 3 mM NaHCO₃, pH 9-6, 1 mM MgCl₂).

**Bioinformatic analysis.** Comparisons of nucleotide sequences and deduced amino acid sequences with the nonredundant GenBank, EMBL, DDBJ, SWISS-PROT and PDB databases were done using the NCBI programs BLASTN, BLASTX and BLASTP (Altschul et al., 1990). Promoter regions were predicted using the promoter predictor program available on the web site http://www.fruitfly.org/seq_tools/promoter.html, selecting prokaryote as the type of organism. For the antigenicity-immunogenicity analysis of amino acid sequences, we used standard methods to locate the most antigenic region based on the hydrophilicity scores and the charged amino acid content (http://www.expasy.ch/cgi-bin/protscalE.html) (Bairoch et al., 1995). Further investigations of secondary and tertiary protein structures were performed, including coiled-coil analysis (Lupas et al., 1991) ([http://www.ch.embnet.org/software/COILS_form.html](http://www.ch.embnet.org/software/COILS_form.html)), a method for prediction of transmembrane domains (Hofmann & Stoffel, 1993; Reithmeier, 1995) to reveal potential exposed domains of peptides, and a program for prediction of signal sequences (Nielsen et al., 1997) ([http://www.ch.embnet.org/software/TMPRED_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)).

**Expression and purification of His-tailed LppS.** Primers 2EN-term and 2EC-term (Table 1) containing *Hind*III and *Nof* restriction endonuclease cleavage sites, respectively, and genomic DNA of strain HRC/581T were used to amplify the 5'-terminal part of the *lppS* gene, corresponding to aa 275 to 4160. This region is orthologous to the *Mycoplasma hypopneumoniae* adhesin-like protein P146 and was predicted to be strongly antigenic. It does not contain TGATGA codons. Plasmid pFFLpSp-His, encoding the polyhistidine-tailed N-terminal part of LppS, was constructed by ligating the amplicon between the *Hind*III and *Not* sites of the T7 promoter-based expression vector pETHIS-1 (Schaller et al., 1999). The plasmid was purified using the QiAprep Spin Plasmid kit (Qiagen), sequenced using primers complementary to the T7 promoter and to the 3'-terminal region flanking the multi-cloning site of the vector to confirm that it contained the expected insert, and used to transform *E. coli* BL21(DE3) cells (Novagen) for expression. A positive clone was inoculated into 50 ml LB broth containing 50 μg ampicillin ml⁻¹ and incubated at 37 °C to an OD₅₄₀ of 0-45. Expression was induced by addition of 1 mM IPTG and incubation continued for another 3 h. The cells were sedimented by centrifugation at 3000 g for 10 min, resuspended in 5 ml PN buffer (50 mM NaH₂PO₄, pH 8-0, 300 mM NaCl), sonicated with a microtip for 4 min with the power output control at 7 and a duty cycle of 50 % (1 s pulse) in a Branson Sonifier 250 (Branson Ultrasonics), and then centrifuged at 15000 g for 20 min. The supernatant containing the cytosolic fraction was kept and the pelleted cell debris was resuspended in 5 ml PN buffer. Analysis of the sonicated fraction on SDS/10 % acrylamide gels (Laemmli, 1970) showed that the induced protein was in the pellet. Guanidine hydrochloride was added to the pelleted cell debris to a final concentration of 6 M and the mixture was loaded onto a prewashed 2-5 ml bed volume Ni/nitrilotriacetic acid/agarose column (Qiagen) and the column washed once with 30 ml PNG buffer (50 mM NaH₂PO₄, pH 8-0, 300 mM NaCl, 6 M guanidine hydrochloride). Step elution of the protein was performed with 10 ml PNG buffer at pH 7-0, 6-0, 5-5, 5-0 and 4-5, collecting 1 ml fractions. The fractions were dialysed and analysed on SDS/10 % acrylamide gels. The purified fusion protein eluted at pH 5-0 was dialysed overnight against PN buffer and was designated LppS-His.

(10 mM Tris/HCl, 1 mM EDTA, 0-8 % NaCl, pH 8-0) and then resuspended in TES buffer to a concentration of approximately 10³ ml⁻¹. 

(E. coli)
Table 1. Primers used in this study

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<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Nucleotide position*</th>
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<td>1391–1413</td>
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*The nucleotide position is based on the sequence of AJ318939.
†Obtained with the Oligonucleotide Properties Calculator at http://www.basic.nwu.edu/biotools/oligocalc.html, using the nearest neighbour method and the parameters 300 nM primer and 50 mM salt (Na+).
‡Lower-case letters indicate nucleotides added to create restriction enzyme recognition sites for cloning.
§Not considering nucleotides added to create the restriction endonuclease recognition sites.

Production of monospecific rabbit anti-LppS antibodies, IgG purification and Fab (antigen-binding fragment) preparation.

Monospecific, polyclonal antibodies directed against LppS were obtained by immunizing a rabbit subcutaneously with 50 μg LppS'-His protein in 500 μl PN buffer mixed with 500 μl adjuvant 10 (GERBU Biotechnik) followed by two booster immunizations, with the same amount of protein and the same adjuvant 3 and 6 weeks later. The animal was bled 8 days after the second booster immunization. Serum was prepared from the blood sample and stored at −20 °C.

Immunoglobulin (IgG) fractions from serum obtained from the rabbit prior to immunization and from monospecific anti-LppS'-His serum were purified using the HiTrap Protein G HP kit (Amersham Pharmacia Biotech) as indicated by the manufacturer. Briefly, HiTrap Protein G HP was swollen and washed extensively with binding buffer (20 mM sodium phosphate, pH 7.0). After the addition of 4 ml serum and extensive washing of the column with binding buffer, the bound IgG was eluted with 20 ml elution buffer (0.1 M glycine/HCl, pH 2.7). The protein in the eluate was precipitated with 6-4 g ammonium sulfate. After 10 min centrifugation at 12 000 g, the pellet was resuspended in 1 ml dialysis buffer (50 mM NaHCO₃, pH 8-0, 125 mM NaCl) and then dialysed at 4 °C overnight against 1000 vols dialysis buffer.

Fab fragments were prepared using the ImmunoPure Fab Preparation kit (Pierce) following the manufacturer’s instructions. Briefly, Fab and Fc (crystallizable fragment) fragments were generated from anti-LppS'-His IgG by incubating 1.7 mg purified IgG with immobilized papain at 37 °C for 5 hr. The crude digest was then applied to a column of immobilized protein A. Separation of 1 mg Fab fragments from the Fc fragments bound to protein A was achieved by washing the column. The Fab fragments were then dialysed overnight against PBS buffer (140 mM NaCl, 2-7 mM KCl, 15 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7-4). Protein concentrations were determined by the method of Bradford (1976).

Hyperimmune rabbit serum directed against M. conjunctivae HRC/581 and sera from chamois free of IKC or with IKC have been described earlier (Belloy et al., 2001; Degiorgis et al., 2000).

Extraction of Tween 20 soluble proteins. Tween 20 solubilization of cells was done as described previously (Belloy et al., 2001). Briefly, M. conjunctivae HRC/581 cells were harvested by centrifugation and then resuspended in TES buffer at 1 mg wet cells ml⁻¹. Subsequently, Tween 20 was added to a final concentration of 1% (v/v). The suspension was then incubated at 37 °C for 90 min with gentle mixing and cleared by ultracentrifugation at 48 000 g at 4 °C for 60 min. The supernatant was then filtered through a low protein-binding membrane (0.2 μm, PALL Gelman Laboratory). This procedure resulted in a fraction of dissolved mycoplasma membrane proteins and was referred to as the Tween 20 membrane protein fraction.

Culture of lamb joint synovial cells. Cell cultures (LSM 192) from lamb carpal joint synovial tissue were prepared in six-well tissue culture plates. The tissue was mechanically minced and subsequently incubated at 37 °C in tissue culture medium (MEM (Biochrom) supplemented with 10% foetal calf serum, 2-5 mM L-glutamine, 100 μM penicillin in ml⁻¹ and 100 μg streptomycin ml⁻¹) in a CO₂ incubator. The medium was changed every 3 days. After reaching confluence, the cells were trypsinized and transferred into 75 cm² tissue culture flasks. The cells were passaged at weekly intervals.
intervals and transferred into 24-well plates. Synovial cells were used in adherence assays when they had reached confluence, with 10^5 cells per 2 cm^2 well. Non-specific binding was blocked by incubation of the cells with a solution of 0.1% BSA for 15 min at 37 °C prior to addition of the mycoplasma suspensions for adherence assays.

**Adherence and inhibition assays.** For adherence assays, *M. conjunctivae* type strain HRC/581^T^ was grown in 200 ml standard mycoplasma culture medium containing 20 μCi (740 kBq)/μCi-[14C] palmitic acid for 3 days at 37 °C with 5% CO₂. The mycoplasma suspensions were washed three times in buffer A (50 mM Tris/HCl, pH 7.2, 100 mM NaCl and 1 mM CaCl₂) to remove unincorporated [14C]palmitic acid. Cultures were frozen at −80 °C in small aliquots until used. *M. conjunctivae* were used immediately after thawing and were not frozen again. In each case, three parallel trials were carried out.

To determine the optimal concentration of mycoplasmas for the adherence assay, dilutions (1:2 to 1:32) of 10^10 c.f.u. ml⁻¹ culture were prepared in buffer A (Sachse, 1998). Two hundred microlitres of each dilution was transferred onto each LSM 192 monolayer and incubated for 2 h at 37 °C. After removing excess liquid, the LSM 192 cells were washed three times with 500 μl buffer A to remove non-adherent mycoplasmas and then solubilized by incubation with 100 μl 1% (w/v) SDS and 500 μl buffer B for 2 h at 37 °C with shaking. The lysed suspension of LSM 192 cells from each well was then transferred into a vial containing 3 ml Emulsifier Scintillator Plus (Packard Instrument Company) and decays per minute were counted using a scintillation spectrometer (Wallac 1410 Liquid Scintillation Counter, Perkin Elmer). Relative adherence (percentage of mycoplasmas attached) was then measured in 200 μl of the original mycoplasma suspension at the corresponding dilution. A pilot experiment with freshly grown and labelled mycoplasma cells showed that adherence was not affected by freezing and thawing.

For inhibition experiments a 1:5000 dilution of purified IgG from rabbit monospecific anti-LppS serum at a concentration of 3.4 mg ml⁻¹, a 1:500 dilution of serum from an uninoculated rabbit at a concentration of 0.38 mg ml⁻¹, as well as dilutions of 1:10, 1:40, 1:100 and 1:200 of the Fab fraction from anti-LppS IgG at a concentration of approximately 0.1 mg ml⁻¹ and from the serum IgG from the rabbit prior to immunization were prepared in buffer A. Each dilution was incubated with the appropriate concentration of 14C-labelled *M. conjunctivae* HRC/581^T^ in a final volume of 200 μl for 2 h at room temperature with shaking. The mycoplasma/antibody mix was then transferred into wells containing LSM 192 cells and incubated for an additional 2 h at 37 °C. Cells were then treated as described above.

To measure the adherence capacity of purified recombinant LppS protein, approximately 25 μg of the LppS-His protein was incubated with LSM 192 cells for 2 h. After three washes, the cells were lysed using a solution of 60 μl 1% (w/v) SDS and 240 μl buffer A. The resulting lysate containing bound LppS-His and disrupted LSM 192 cells was concentrated to 100 μl and mixed with 2× SDS-PAGE sample buffer, boiled for 5 min, proteins separated in 10% polyacrylamide gels using SDS-PAGE and blotted onto nitrocellulose membrane (0.2 μm pore size, Bio-Rad). The membranes were then incubated with the rabbit monospecific anti-LppS-His serum. The specificity of LppS-His binding to LSM 192 cells was assessed by inhibition of binding of LppS-His by different dilutions of purified IgG directed against LppS. Purified IgG from the serum of the rabbit prior to immunization and anti-His antibody (Amersham Pharmacia Biotech) were used as controls.

**RESULTS**

**Cloning, screening and sequencing of antigenic genes of *M. conjunctivae***

A gene library of the genomic DNA of *M. conjunctivae* type strain HRC/581^T^ containing about 10^6 clones ml⁻¹ was constructed in the λ phage ZAP Express vector. Approximately 30 000 recombinant phage clones were screened with a rabbit serum raised against whole cell antigens from *M. conjunctivae*. Positive clones were converted to phagemids. Selected plasmids were then sequenced partially and plasmid pJFF2E, which contained two incomplete ORFs, was retained and sequenced completely. One of the ORFs was represented by its 3′-terminal end. This gene had significant similarity to an adhesin-like lipoprotein (P146) (GenBank accession number AAF91425), the adhesin MHP1 (King et al., 1997), a ciliary adhesin (Wilton et al., 1998) and the adhesin P97 (Hsu et al., 1997), all of *M. hyopneumoniae*.

The entire 3.5 kb insert in plasmid pJFF2E was sequenced in both directions and the DNA primers p2ET71 and p2ET31 derived from its sequence (Table 1) were used in a PCR with genomic DNA of *M. conjunctivae* to confirm the integrity of the clone. The clone pJFF2E was found to contain ORFs encoding the carboxy-terminal part of a putative Ser-rich adhesin-like protein and the amino-terminal part of a putative lipoprotein. Both ORFs were in the same orientation (Fig. 1). Based on this sequence, primers were designed to complete the DNA sequence of both ORFs. These primers were used with the Vectorette System. Five different, complementary Vectorette libraries were made. Only PCR products between approximately 400 and 3000 bp were amplified and sequenced. Based on each newly sequenced fragment, new primers were designed and novel Vectorette-based amplifications were performed until a DNA segment of 7693 bp, including the complete ORF for each of the two genes was sequenced. This segment contained two genes encoding predicted lipoproteins. The protein of 1405 aa (152 kDa) was named LppS and the protein of 947 aa (105 kDa) was named LppT. Upstream of the ORF encoding LppS, two putative −35 and two putative −10 boxes were found (Fig. 1). In addition, putative ribosome-binding sites were found upstream of the ATG initiation codon of the two ORFs. The two ORFs with their promoter region were flanked by sequences predicted to form mRNA structures very similar to rho-independent transcriptional stop signals (Fig. 1). The calculated free energy was −3.5 kcal mol⁻¹ for the hairpin preceding the promoter region and −13.5 kcal mol⁻¹ for the hairpin downstream of the two complete ORFs. Southern blot analysis of HindIII-digested genomic DNA of *M. conjunctivae* HRC/581^T^ using the LppS gene probe detected a single copy of the LppS gene (Fig. 2).

**Characterization of LppS**

The largest ORF, encoding the LppS protein, which was predicted to contain 1405 aa and have a molecular mass of
152 kDa, contained a typical prokaryotic signal peptidase cleavage site at residue 27. The leader sequence was predicted to have a typical transmembrane helix structure, with a significant inside-to-outside helix formation score of 2306 on the TM prediction scale. These characteristics suggest that LppS is a lipoprotein. It contained a prominent polyserine region towards its C-terminal end and hence was designated LppS. The coding sequence contained only one TGATrp codon, at amino acid position 104. Sequence similarity analysis of the nucleotide and amino acid sequences deduced from \textit{lppS}, using the SWISS-PROT, EMBL and GenBank sequence databases, confirmed the similarity between LppS and the adhesin like-protein P146, adhesin MHP1, ciliary adhesin and adhesin P97 of \textit{M. hyopneumoniae}. The highest similarity was in the first 383 aa with P146, where 22 % identical and 43 % similar residues were found. Between aa 611 and 1079, the sequences had 21 % identity and 36 % similarity. The sequences of the other three adhesins; MHP1, the ciliary adhesin and P97, had 24 % identity and 42 % similarity between positions 24 and 375. The C-terminal end of LppS had two unusual characteristics: a serine-rich segment, with 37 serine residues out of 41 aa (Fig. 1) between residues 1297 and 1338 and following this, from aa 1339 to 1383, a proline-rich region with 18 proline residues out of 44 aa. This C-terminal region had no similarity to any of the sequences in the EMBL/GenBank and SWISS-PROT databases.

Immunoblots containing \textit{M. conjunctivae} HRC/581\textsuperscript{T} membrane protein antigens prepared by extraction with the neutral detergent Tween 20 were reacted with monospecific polyclonal antibodies directed against the recombinant LppS\textsuperscript{9}-His peptide. The antibodies bound to two bands of 150 kDa in the detergent-associated fraction, confirming that LppS was a membrane protein (Fig. 3). The doublet band may be due to the presence of both unprocessed pre-LppS and mature LppS in the fraction. Rabbit anti-\textit{M. conjunctivae} hyperimmune antiserum and serum from a chamois that had been infected with \textit{M. conjunctivae} also detected the distinct doublet of 150 kDa, but at a weaker intensity (Fig. 3a). Sera from sheep with IKC reacted strongly with purified LppS\textsuperscript{9}-His in Western blots, while sera from IKC-free sheep did not react (Fig. 3b).

**Adhesion**

As the amino acid sequence of LppS was similar to those of the \textit{M. hyopneumoniae} adhesin-like protein P146, adhesin MHP1, ciliary adhesin and adhesin P97, we studied the role of LppS in adhesion of \textit{M. conjunctivae}. Adhesion assays were performed with the monolayer lamb cell culture LSM
Two adhesion assays were developed, the first using purified recombinant LppS\(^9\)-His protein and the second using radioactively labelled cultures of \textit{M. conjunctivae} HRC/581\(^T\). As shown in the immunoblot (Fig. 4, lane A) LppS\(^9\)-His showed an affinity for the LSM 192 culture. This affinity was completely inhibited when the LppS\(^9\)-His protein was incubated with purified rabbit anti-LppS IgG (Fig. 4, lane B). When LppS-His was incubated with IgG purified from serum collected from the rabbit prior to immunization there was no inhibition of adhesion of LppS\(^9\)-His to the LSM 192 cells (Fig. 4, lane C). Incubation with a monoclonal mouse anti-polyHis antibody did not inhibit adhesion, indicating that adhesion was not influenced by the polyhistidine tail of the recombinant LppS\(^9\)-His protein (Fig. 4, lane D).

To determine the role of LppS in adhesion of \textit{M. conjunctivae}, an assay for adhesion of \textit{M. conjunctivae} HRC/581\(^T\) cells to lamb synovial membrane cells was developed. The proportion of \(^{14}\text{C}\)-labelled \textit{M. conjunctivae} adhering to LSM 192 cells increased with decreasing concentration of the mycoplasmas (Fig. 5). Purified Fab fragments obtained from monospecific anti-LppS IgG significantly reduced adhesion (Fig. 6). Fab fragments from IgG purified from serum collected from the rabbit prior to immunization caused only a background level of 6-7% reduction in the amount of \textit{M. conjunctivae} adhering to LSM 192 cells (Fig. 6).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Detection of lppS in \textit{M. conjunctivae} strain HRC/581\(^T\). Southern blot of \textit{HindIII}-digested genomic DNA from strain HRC/581\(^T\) hybridized with a digoxigenin-labelled probe for the lppS gene generated with primers Ser_start2 and Ser_end (Table 1).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Immunodetection of LppS. A. Immunoblot strips containing the Tween 20 fraction of \textit{M. conjunctivae} HRC/581\(^T\) detected with different sera. Lanes: 1, serum from chamois free of IKC diluted 1/50 (negative control); 2, serum from a chamois with IKC from a natural outbreak diluted 1/50 (positive control); 3, rabbit serum raised against whole \textit{M. conjunctivae} HRC/581\(^T\) cells (diluted 1/2000); 4, rabbit serum raised against LppS\(^9\)-His (diluted 1/2000). B. Immunoblot strips containing purified recombinant LppS\(^9\)-His protein reacted with sheep sera diluted 1/50. Sheep numbers 59, 61, 63, 65 and 62 were from natural IKC outbreaks; sheep numbers 2812, 2815, 2819, 2817 and 2813 were free of IKC. Each sheep serum was tested for antibodies against \textit{M. conjunctivae} by ELISA as previously described (Belloy \textit{et al.}, 2001). ELISA values from the serological diagnosis for IKC of these animals (Belloy \textit{et al.}, 2001) are indicated below the immunoblot strips.}
\end{figure}

\section*{DISCUSSION}
Analysis of a subset of clones from an expression gene library of \textit{M. conjunctivae} strain HRC/581\(^T\) revealed a predicted bicistronic operon with the two genes lppS and lppT encoding potential membrane proteins. The lppS gene and its gene product LppS were analysed in detail as they showed...
significant sequence similarity to the adhesin-like protein P146, adhesin MHP1, a ciliary adhesin and adhesin P97 of *M. hyopneumoniae*, and to a lesser extent to other adhesin proteins. The most striking feature of the amino acid sequence of LppS was the C-terminal end, which contained a stretch of 37 serine residues interrupted at only two sites by alanine-glycine after 5 and after 4 serine residues, leaving a contiguous 27 polyserine segment. The sequence of this part of the *lppS* gene was verified by sequencing PCR amplified DNA segments from genomic DNA (data not shown). No protein with such a polyserine segment has yet been described. The function of this domain is unclear. However, LppS has some sequence similarity to the fibrinogen binding protein, clumping factor (ClfA) of *Staphylococcus aureus* (McDevitt *et al.*, 1994), which has a repeated serine-aspartate dipeptide domain.

Adhesion of recombinant LppS'-His to lamb synovial membrane cells and inhibition of adhesion by monospecific, polyclonal antibodies directed against LppS showed that LppS could adhere specifically to ovine cells. This adhesion was not influenced by the poly-His tail on the recombinant protein. The lamb synovial membrane cells were chosen as sheep are the natural host of *M. conjunctivae*. Cells from passage 4–15 were used, as higher passages of these cells were known to show a reduced capacity to bind other mycoplasma species that are pathogenic in *Caprinae*. Adhesion experiments with radioactively labelled *M. conjunctivae* and inhibition experiments using Fab fragments from IgG directed against LppS revealed that LppS was involved in adhesion of *M. conjunctivae* to sheep cells. The inhibition of adhesion by Fab fragments of anti-LppS IgGs was up to 80% at a dilution of 1 : 10, and diminished with increasing serum dilutions. Fab fragments derived from the serum of the rabbit prior to immunization were only able to inhibit 6-7% of the adhesion. Hence, we consider LppS to be an adhesion protein of *M. conjunctivae*.

**Fig. 4.** Adhesion of LppS'-His to LSM 192 cells. The immuno-blot was reacted with monospecific polyclonal anti-LppS'-His antibodies to detect the presence of bound recombinant LppS'-His. A, LppS'-His added directly to LSM 192 cells; B, LppS'-His incubated with anti-LppS'-His IgG prior to application to LSM 192 cells; C, LppS'-His incubated with IgG from the unimmunized rabbit prior to application to LSM 192 cells; D, LppS'-His incubated with anti-His monoclonal antibodies prior to application to LSM 192 cells.

**Fig. 5.** Adherence assay. Histogram representing the relative adherence of serial dilutions of 14C-labelled *M. conjunctivae* HRC/581T to lamb synovial cells LSM 192 in culture after incubation at 37°C. Values below the columns indicate the dilutions. The data are the mean of three independent measurements. Error bars, SEM.

**Fig. 6.** Inhibition of adherence. Adhesion of 14C-labelled *M. conjunctivae* HRC/581T to LSM 192 cells was assessed after incubation with different sera. The inhibition was then calculated as the reduction of adhesion caused by the presence of the serum compared to adhesion without treatment. The bars indicate the percentage of inhibition of adhesion in the presence of: Fab fragments from anti-LppS IgGs diluted 1 : 10, 1 : 40, 1 : 100 and 1 : 200 in buffer A (first four bars from left to right); Fab fragments purified from IgG from the serum of the rabbit prior to immunization (preserum); or buffer only. The data are the mean of three independent measurements. Error bars, SEM.
Serological analysis revealed that chamois and sheep that had suffered from IKC had immunological reactions to LppS, while animals from areas without IKC did not have anti-LppS antibodies. The second gene, lppT, in the same operon, encoded a protein of 947 aa with a calculated molecular mass 105 kDa. This gene product was not investigated further in this study. However, sequence data indicate that the gene product encoded by lppT is also a membrane protein with a signal sequence of 34 aa at the amino-terminal end, followed by two transmembrane structures. It shows significant similarity to the membrane proteins P76 and P110 of M. hyopneumoniae.

LppS and LppT.

In summary, we developed a binding assay and showed that the protein encoded by the lppS gene was strongly implicated in the adhesion of M. conjunctivae to its host. Hence, LppS is likely to play an important role in mycoplasma-host cell interactions and can be considered a virulence factor of M. conjunctivae.

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