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Molecular Cloning, Expression, and DNA Sequence Analysis of the Gene That Encodes the 16-Kilodalton Outer Membrane Lipoprotein of *Serpulina hyodysenteriae*

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The gene (*smpA*) that encodes the 16-kDa outer membrane lipoprotein of *Serpulina hyodysenteriae* was cloned in *Escherichia coli*, and its primary structure was determined by nucleotide sequencing. The putative open reading frame encodes a prolipoprotein of 16.8 kDa which in its fully acylated and cleaved form is 15.1 kDa. Analysis of the N-terminal amino acid sequence derived from the DNA sequence revealed the presence of a signal sequence and a putative acylation and signal peptidase II cleavage site (Phe-Ala-Val-Ser-Cys). In *E. coli*, processing of the prolipoprotein was less efficient than that observed in *S. hyodysenteriae*, and globomycin, an inhibitor of signal peptidase II, inhibited cleavage of the lipoprotein expressed in *E. coli* but did not inhibit cleavage in *S. hyodysenteriae*.

When *Serpulina hyodysenteriae* is inoculated into conventionally reared pigs, clinical signs of dysentery consisting of mucohemorrhagic diarrhea are produced (12, 33). Attempts have been made to develop vaccines against infection by *S. hyodysenteriae* which have been based on whole-cell bacterins (11, 15, 21, 23) and oral vaccination with attenuated strains of the spirochaete given either alone (16) or together with a parenterally administered bacterin (15). These vaccines have provided some degree of protection from experimental oral challenge with *S. hyodysenteriae* (36) and in challenge experiments using colonic intestinal loops in pigs (18, 19). However, protection was serotype specific and probably relied upon an immune response to endotoxin or lipopolysaccharide components.

Antigens other than lipopolysaccharide may reside in the outer membrane or endoflagella of *S. hyodysenteriae* and may stimulate protective immune responses. Our previous investigations have demonstrated immune responses to the endoflagellum polypeptides of 29 to 44 kDa (20) and also to a 16-kDa membrane-associated polypeptide which was common to strains of *S. hyodysenteriae* (27). This antigen has been identified as an outer membrane lipoprotein which is exposed on the surface of the spirochete (34, 35). Both polyclonal (27) and monoclonal antibodies (MAbs) (34) to this antigen inhibited the growth of *S. hyodysenteriae* strains in vitro. Therefore, protective immune responses may be stimulated by this antigen, and this report concerns the molecular cloning, DNA sequence analysis, and expression of the gene that encodes this lipoprotein in *Escherichia coli*.

Cloning strategy. High-molecular-weight genomic DNA was prepared from *S. hyodysenteriae* P18A (28). The DNA was partially digested with *Sau*3a and size fractionated on a 10 to 40% (wt/vol) sucrose gradient at 20,000 × *g* for 18 h. The 2- to 6-kbp fraction was partially end filled with nucleotides G and A by using DNA polymerase (Klenow). The λZAP II vector (Stratagene, La Jolla, Calif.) was prepared by digestion with *Xho*I, and the single-stranded ends were partially end filled with nucleotides T and C. After ligation of genomic DNA and the vector, the DNA was packaged in vitro by using packaging extracts prepared from *E. coli* BHB2690 (N205 *recA* [λ*imm*434 c*Its b*2 *red D*am *S*am/λ]) and BHB2688 (N205 *recA* [λ*imm*434 c*Its b*2 *red E*am *S*am/λ]) and the recombinant bacteriophage were stored in SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$, 0.01% [wt/vol] gelatin in 50 mM Tris/HCl, pH 7.5) containing 2% (vol/vol) chloroform. *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q} *lacZ*Δ*M* Tn*10 tet*]) was transfected with the phage stock and plated in top agar containing 2.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and 4 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml for determination of the ratio of blue and white plaques and, hence, the number of recombinant phage. Recombinant phage (31,000) were screened by the plaque lift assay for expression of the 16-kDa antigen by using B50 hyperimmune antiserum and ^{125}I-labelled protein A (Amersham). Five positive clones, which were also positive with MAb F325 AC4, were subcloned into phagemid pBluescript SK$^-$ by excision of the plasmid from the λ vector with the R408 (f1) helper phage by the Stratagene protocol. Excision of the recombinant plasmids from the λZAP II recombinant phage resulted in production of five plasmids (designated pWT350, pWT640, pWT734, pWT760, and pWT850), which contained inserts of 4.9, 2.0, 1.4, 1.4, and 3.5 kbp and were stable in *E. coli* XL1-Blue (Fig. 1). Expression of the antigen was detected with MAb F325 AC4, and the antigen was expressed by all of the clones (data not shown). Plasmid pWT734 was selected for further analysis because this plasmid contained the smallest DNA insert which expressed the polypeptide.

Generation of deletions and DNA sequence analysis. DNA from plasmid pWT734 was digested with restriction enzymes *Pst*I and *Hin*dIII. This provided both 3′ and 5′ overhangs within the vector DNA to the left of the DNA insert. A number of deletions were made with the Erase-a-Base kit (Promega, Madison, Wis.) from the 5′ overhang into the insert DNA by digestion with exonuclease III at timed intervals, followed by S1 nuclease digestion to provide blunt ends for religation of the plasmid DNA. Nucleotide sequencing was performed by the dideoxy-chain termination method

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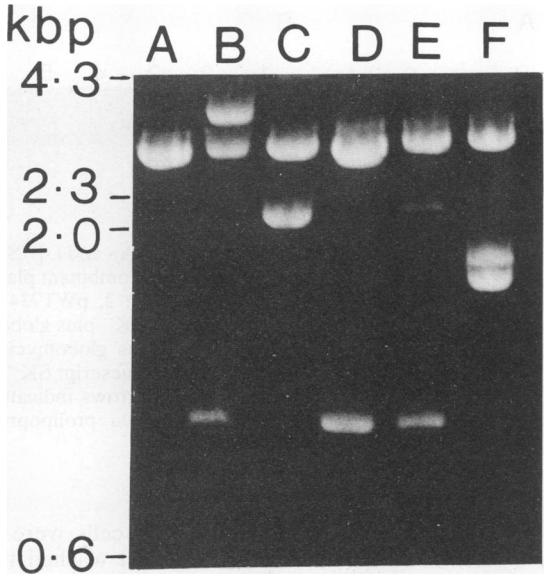

FIG. 1. Agarose gel electrophoresis of restriction endonuclease digestion of recombinant plasmids that encode the 16-kDa prolipoprotein. Lanes: A, pBluescript SK$^-$; B, pWT350; C, pWT640; D, pWT734; E, pWT760; F, pWT850. Plasmids were digested with *Kpn*I and *Hinc*II. Molecular size markers are indicated.

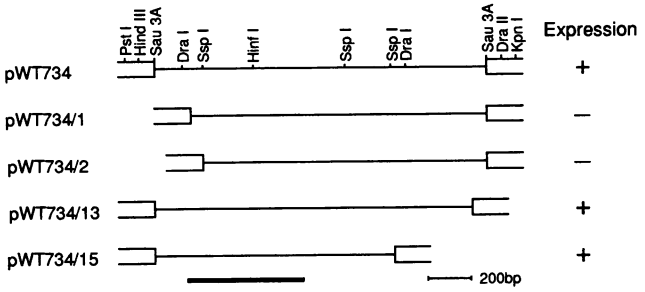

FIG. 2. Restriction endonuclease map of recombinant plasmid pWT734, the deletions constructed, and expression of the 16-kDa lipoprotein. Open boxes indicate pBluescript SK$^-$ vector DNA, and the thick line indicates the position of the open reading frame.

of Sanger et al. (25) with the modified T7 DNA polymerase (Sequenase) kit (United States Biochemical, Cleveland, Ohio). Single-stranded template DNA was prepared from plasmid pWT734 by the method of Murphy and Kavanagh (22).

Computer programs (29) were used to compile sequence data obtained from sequencing gels, and the University Wisconsin-Madison Sequence Analysis Software Package of the Genetics Computer Group Inc., version 7, was used to analyze the sequence data (9) on a MicroVAX 3600. Sequence data for most of the insert were obtained by using only the universal and reverse M13 sequencing primers and oligonucleotides 5′-CATTTAAAGGAGTTCAAAA-3′, 5′-TTATTAAAGTTGCTCTGTTTA-3′, and 5′-TAGTTGTGT TAGGTGCAAT-3′, which were synthesized with a 381A Applied Biosystems oligonucleotide synthesizer. The sequence data also provided details of the extents of the deletions generated. Western blot (immunoblot) analysis, with MAb F325 AC4, of *E. coli* XL1-Blue containing the truncated plasmid indicated that when the first 143 bases were removed from the insert DNA (pWT734/1), there was no expression of the antigen; this region is thus important for expression of the gene (Fig. 2). An additional deletion of 166 bp from the 5′ overhang (pWT734/2) was also negative by Western blotting. However, deletions of 30 (pWT734/13) and 400 (pWT734/15) bases, made from the opposite end of the insert by digestion with *Kpn*I, to provide a 3′ overhang; *Dra*II, which leaves a 5′ overhang; and exonuclease III did not affect expression of the antigen.

Sequence analysis revealed a potential open reading frame of 477 bases from 119 to 595 (Fig. 3), which starts with a methionine and ends with a TAA stop codon. The predicted mature protein is 159 amino acids long with a molecular size of 16,819 daltons. The theoretical size (predicted from the DNA sequence) of the partially acylated, uncleaved prolipoprotein is 17.4 kDa, and that of the cleaved, fully acylated lipoprotein is 15.1 kDa (assuming that palmitic acid is the only fatty acid). These are slightly lower figures than those obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18 and 16 kDa, respectively).

A potential ribosome-binding site (AGGAG) from −13 to −9 (bases 106 to 110) exists, and putative −10 (TATTAT, bases 87 to 92) and −35 (TTCAAA, bases 61 to 66) promoter regions were also identified. As the 16-kDa antigen of *S. hyodysenteriae* appeared from previous investigations to be a lipoprotein, we identified a signal sequence with positively charged amino acids at positions 1, 2, and 3; a core of hydrophobic amino acids; and a cysteine residue which

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  GATCAAATTGTTCTTAAATAATACCATAATCAAAAAATATAAATGTTTC - 49
  Sau3A
            -35                              -10
TAAAACAATTTTTCAAATAAGCATTAGAAATAATGTTTATTATAAATTTCA - 100

      S/D
TTTAAAGGAGTTCAAAATATGAACAAAAAAATTTTCACACTATTTTTAGTA - 151
                  M  N  K  K  I  F  T  L  F  L  V

GTTGCCGCATCTGCAATATTTGCAGTAAGCTGTAACAACAAAACAACAAAT - 202
 V  A  A  S  A  I  F  A  V  S  C  N  N  K  T  T  N

CCTACTTCAAACAGCTCAGAAAAAAAGATTGTTACAGAAGAAGATTTTAAA - 253
 P  T  S  N  S  S  E  K  K  I  V  T  E  E  D  F  K

AATGCTATAGAAGGTTTAACTTACAAAACTTGGGCATTTACTGGAAAAGGA - 304
 N  A  I  E  G  L  T  Y  K  T  W  A  F  T  G  K  G

AAATCATTTAATTTCGGAAGTCCTATAACTGTAGAGGCTACATCTGGTTCT - 355
 K  S  F  N  F  G  S  P  I  T  V  E  A  T  S  G  S

GATTCATTAGCAGGTGTAGAAAAGGGATTTGGAAATGCATTAAAATCAGCT - 406
 D  S  L  A  G  V  E  K  G  F  G  N  A  L  K  S  A

TTAGCAGCTAAAGGAATTGATACTGGAAATATAACTTTTGATAAAGGTGGA - 457
 L  A  A  K  G  I  D  T  G  N  I  T  F  D  K  G  G

GCAAGTTCTTCAGATAAAACATCTGTATCTTTCAAATTTACTCCTAAAGCT - 508
 A  S  S  S  D  K  T  S  V  S  F  K  F  T  P  K  A

CTTGGAACATCAAATTTTGAAGAAAAATTAAAATCTTCTGTAAAAGAAGTA - 559
 L  G  T  S  N  F  E  E  K  L  K  S  S  V  K  E  V

GAAATAAAATTAACTCCTAAAGAAAATTGGGGAGCTTAATTAAAATTGACA - 610
 E  I  K  L  T  P  K  E  N  W  G  A  ***
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FIG. 3. Nucleotide sequence of the *smpA* gene of *S. hyodysenteriae*, which encodes the 16-kDa prolipoprotein from the 5′ end of the insert to just downstream of the open reading frame. The deduced amino acid sequence is presented in one-letter code. The asterisks indicate a stop codon. Putative −35 and −10 promoter regions and a ribosome-binding site (S/D) are underlined.

would be a possible acylation site. In the open reading frame identified, there is one cysteine only at amino acid position 22 from the start of the open reading frame, which is part of the sequence Phe-Ala-Val-Ser-Cys, a potential signal peptidase II cleavage site. The consensus sequence (occurring in approximately 75% of the cases) for this peptide is Leu-(Ala, Ser)-(Gly, Ala)-Cys (13). The peptide sequence of the *S. hyodysenteriae* 16-kDa lipoprotein deviates from this consensus. There is a Ser at position −1, which is favored in only 8% of the cases, a Val at −2 (5%), an Ala at −3 (8%), and a Phe at −4 (4%). However, this peptide is similar to *Mycoplasma hyorhinis* lipoprotein P37 (Phe-Ala-Ile-Ser-Cys), which has Ile instead of Val at −2 (10), and the 34-kDa lipoprotein (TmpD) of *Treponema pallidum*, which has a cleavage site consisting of four amino acids (Phe-Ser-Ala-Cys) (26, 32).

Another important criterion for efficient prolipoprotein modification appears to be the presence of a β-turn secondary structure immediately following the Cys residue (13), and mutants with a decreased probability for β-turn structures have been shown to be defective in prolipoprotein modification (17). Computer prediction (7) of the amino acid secondary structure suggests that β-turn secondary structures may occur after the cysteine putative acylation site in a six-amino-acid peptide which contains the two asparagine residues at positions +2 and +3.

Expression studies. Minicells containing plasmids were prepared by the method of Reeve (24) as modified by Boronat et al. (3). Expression of plasmid-encoded polypeptides was identified by in vivo labelling with either L-[^{35}S]methionine or L-[^{35}S]cysteine (Amersham) at a concentration of 200 μCi/ml for 1 h at 37°C, and separation of the labelled polypeptides was performed by SDS-PAGE. The gel was dried, and autoradiography was performed after fluorographic enhancement (35). Inhibition of processing of the lipoprotein in both the spirochete and *E. coli* was performed by incubation of (i) *S. hyodysenteriae* VS1 in tryptic soy broth for 12 h and (ii) the *E. coli* clone possessing plasmid pWT734 in Luria-Bertani broth for 2 h with globomycin (a gift of M. Inukai, Fermentation Research Laboratories, Sankyo Company Ltd., Tokyo, Japan) at a concentration of 200 μg/ml. As the globomycin was dissolved in 2% (vol/vol) ethanol, a control *E. coli* culture containing 2% (vol/vol) ethanol was also included in the minicell studies.

Minicell analysis of the gene products of plasmid pWT734 and the effect of globomycin on posttranslational modification are shown in Fig. 4. When L-[^{35}S]methionine was used to label the gene products, only the larger, uncleaved prolipoprotein of 18 kDa was labelled. This was unaffected by the presence of globomycin at 200 μg/ml. The demonstration of the 18-kDa spirochete prolipoprotein only was unexpected until DNA sequence data revealed the presence of only one methionine residue at the N terminus of the prolipoprotein, which would be lost when the signal sequence was cleaved. Therefore, to demonstrate the inhibition of cleavage of the signal sequence, labelling of minicells was performed with L-[^{35}S]cysteine. Both cleaved and uncleaved forms of the lipoprotein were present, and globomycin appeared to inhibit cleavage (Fig. 4B). The presence of 2% (vol/vol) ethanol in the medium (the same concentration as that required to dissolve globomycin) failed to inhibit cleavage of the lipoprotein although processing was reduced.

[^{3}H]palmitate labelling of lipid-modified polypeptides in the *E. coli* clones and also in *S. hyodysenteriae* VS1 was performed as described previously (35). The duration of incubation of [^{3}H]palmitic acid with *E. coli* was 5 h, and that

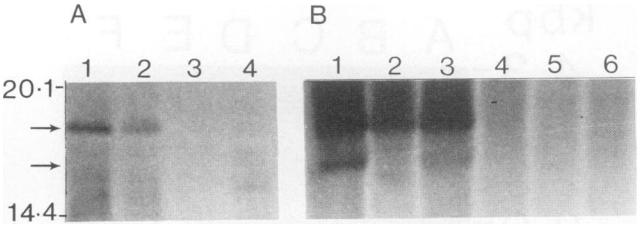

FIG. 4. Autoradiograph of L-[^{35}S]methionine (A)- and L-[^{35}S]cysteine (B)-labelled polypeptides expressed from recombinant plasmid pWT734 in minicells. Panel A lanes: 1, pWT734; 2, pWT734 plus globomycin; 3, pBluescript SK$^-$; 4, pBluescript SK$^-$ plus globomycin. Panel B lanes: 1, pWT734; 2, pWT734 plus globomycin; 3, pWT734 plus ethanol; 4, pBluescript SK$^-$; 5, pBluescript SK$^-$ plus globomycin; 6, pBluescript SK$^-$ plus ethanol. Arrows indicate the positions of the 16-kDa lipoprotein and 18-kDa prolipoprotein bands. Molecular size markers are indicated.

with *S. hyodysenteriae* was 8 h. Labelled cells were collected by centrifugation at 11,600 × *g* and washed three times in phosphate-buffered saline. The labelled components were separated by SDS-PAGE, the gel was dried, and autoradiography was performed after fluorographic enhancement. *E. coli* XL1-Blue containing recombinant plasmid pWT734 was grown in the presence of [^{3}H]palmitic acid, and the 16-kDa lipoprotein was labelled and appeared to be the same size as the [^{3}H]palmitate-labelled lipoprotein of *S. hyodysenteriae* VS1 (Fig. 5). The uncleaved prolipoprotein was not visible. When the *E. coli* strain was grown in the presence of both [^{3}H]palmitic acid and globomycin, there was no labelling of a band at 16 or 18 kDa. In contrast, when *S. hyodysenteriae* VS1 was grown in the presence of both [^{3}H]palmitic acid and globomycin, there was no inhibition of [^{3}H]palmitate labelling and a labelled band of 16 kDa was observed.

Expression of the gene that encodes the lipoprotein in *E. coli* was investigated by Western blotting with MAb F325 AC4. Two bands, of 16 and 18 kDa, were clearly identified in

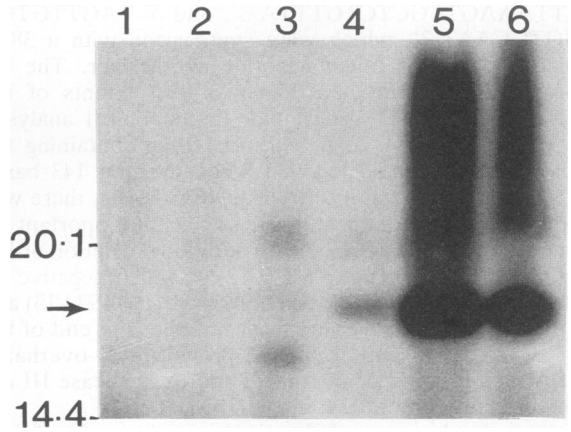

FIG. 5. Autoradiography of [^{3}H]palmitate-labelled components of *E. coli* cells containing recombinant plasmids grown with or without globomycin and separated by SDS-PAGE. pBluescript SK$^-$ plus globomycin (lane 1), pBluescript SK$^-$ (lane 2), pWT734 plus globomycin (lane 3), pWT734 (lane 4), *S. hyodysenteriae* VS1 plus globomycin (lane 5), and VS1 (lane 6) were used. The arrow indicates the position of the 16-kDa lipoprotein band. Molecular size markers are indicated.

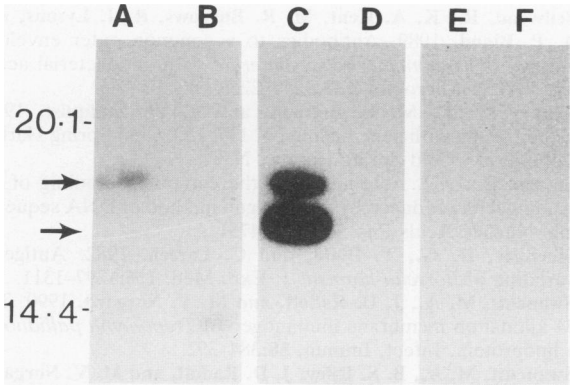

FIG. 6. Western blot of *E. coli* cells containing recombinant plasmid pWT734 (lanes A, C, and E) and pBluescript SK$^-$ (lanes B, D, and F), which were fractionated into periplasmic (lanes A and B), membrane (lanes C and D), and cytoplasmic (lanes E and F) fractions. Arrows indicate the positions of the 16-kDa lipoprotein and 18-kDa prolipoprotein bands. Molecular size markers are indicated.

early-log-phase cultures, but in stationary-phase cultures the predominant form was the fully processed lipoprotein (data not shown). *E. coli* cells containing plasmid pWT734 were fractionated into outer membrane, cytoplasmic membrane, and periplasmic fractions (3), and the components were analyzed by SDS-PAGE and Western blotting. The lipoprotein in both processed and unprocessed forms was associated predominantly with the membrane fraction (Fig. 6).

These results suggest that processing of the lipoprotein in *E. coli* was not as efficient as that in *S. hyodysenteriae*, resulting, particularly in early-log-phase cultures, in a proportion of the uncleaved form. However, globomycin totally inhibited cleavage of the signal sequence in *E. coli*, as shown by L-[^{35}S]cysteine labelling of minicells, but was ineffective in blocking cleavage in *S. hyodysenteriae*. In similar investigations with lipoproteins of other spirochetes, globomycin inhibition experiments have usually been performed with *E. coli* possessing the cloned lipoprotein gene in question and not with the spirochete (14, 26, 31). Either the difficulty in culturing spirochetes in vitro, as is the case with *T. pallidum*, or the fact that globomycin may not be transported efficiently across spirochete membranes could have been a contributing factor. In addition, the specificity or efficiency of the signal peptidases in *E. coli* and *S. hyodysenteriae* may differ because both prolipoprotein and the mature lipoprotein were apparent only in *E. coli*.

It is proposed that this outer membrane lipoprotein of *S. hyodysenteriae* be designated SmpA (*Serpulina* membrane protein A) and that the gene that encodes it be designated, *smpA*. *T. pallidum* also possesses membrane-located lipoproteins (5, 6, 31), although these lipoproteins do not appear to be present on the surface of the spirochete (8). On the other hand, the variable lipoproteins (Vmp) of *Borrelia hermsii* are surface orientated and confer the property of antigenic variation on the organism, thus enabling the organism to evade the immune mechanisms of the host (2, 30). Also, OspA and OspB surface lipoproteins of *B. burgdorferi* (4), which determine the serotype specificity of the spirochetes, also probably play a role in antigenic variation (1). The function of this, apparently abundant, lipoprotein on the surface of the spirochete remains unknown, and there is no evidence to suggest that antigenic variation occurs with *S. hyodysenteriae*. However, the reasons for the persistent nature of swine dysentery could be analogous to those that are being elucidated for relapsing fever or the recurrent Lyme disease.

Nucleotide sequence accession number. The nucleotide sequence data presented here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession no. X68401.

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