INFECTION AND IMMUNITY, Jan. 1995, p. 142–148
0019-9567/95/$04.00+0

Vol. 63, No. 1

Isolation of Extracytoplasmic Proteins from *Serpulina hyodysenteriae* B204 and Molecular Cloning of the *flaB1* Gene Encoding a 38-Kilodalton Flagellar Protein

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Received 11 July 1994/Returned for modification 23 August 1994/Accepted 13 October 1994

Extracytoplasmic proteins were released from *Serpulina* (*Treponema*) *hyodysenteriae* (strain B204) by treatment of whole cells with a nonionic detergent (Tween 20). Centrifugation of the Tween 20-released proteins at 100,000 × *g* sedimented 10 major extracytoplasmic proteins with approximate molecular masses of 44, 43.5, 42, 39, 38, 34, 33.5, 33, 31, and 29 kDa. Treatment of the sedimented fraction with 6 M urea solubilized all of the proteins except the 39-kDa protein. Peptide sequences were obtained for the purified 42-, 39-, 38-, 34-, 31-, and 29-kDa proteins. The peptide sequences of the 42-, 38-, and 31-kDa proteins indicate that they likely are components of the periplasmic flagella. The amino-terminal peptide sequence of the 38-kDa protein was used to design an oligonucleotide probe and to clone an *S. hyodysenteriae* DNA fragment containing the gene encoding this protein. The predicted 290-amino-acid protein sequence derived from the cloned gene was highly homologous to those of several other bacterial flagellar proteins and is preceded by consensus σ^D nucleotide sequences found upstream of other flagellar genes. On the basis of its similarity to the FlaB proteins of other spirochetes, we propose to designate the cloned *S. hyodysenteriae* gene *flaB1* and its encoded protein FlaB1. Vaccination of pigs with FlaB1 or its recombinant counterpart did not protect them from an experimental challenge.

Serpulina (*Treponema*) *hyodysenteriae* is the etiologic agent of swine dysentery (8, 32). Infected animals are afflicted by severe mucohemorrhagic diarrhea leading to dehydration, weight loss, and, in extreme cases, death. Currently available commercial bacterin vaccines, while partially protective, do not provide resistance comparable to that seen in animals that have recovered from infection (13).

Numerous investigators have used a variety of methods to isolate and characterize complex mixtures of antigens from *S. hyodysenteriae* which may play a role in the protective immune response of an animal that has recovered from infection (11, 15, 17, 21, 25, 30, 31, 34, 37). These antigens include proteins found in the outer membrane and periplasmic space, including flagellar proteins, which we collectively refer to as extracytoplasmic proteins (ECP). The accessibility of some of these ECP antigens to the humoral, cellular, and secretory immune responses of the infected pig makes them attractive candidates for evaluation as vaccine components. Vaccination of swine with one of these complex antigen preparations has been reported to reduce the incidence and severity of disease in pigs after an experimental challenge (37). It is not known, however, which antigens in this mixture were responsible for the reported immunity. Identification of specific, immunologically relevant ECP antigens from *S. hyodysenteriae* will likely require the generation, characterization, and evaluation of less complex antigenic mixtures as well as the molecular cloning and heterologous expression of individual genes for these antigens. The cloning of the *flaA1* and *flaB2* genes encoding flagellar sheath and core proteins from *S. hyodysenteriae* and of the *smpA* gene encoding an outer membrane lipoprotein has been recently reported (16, 18, 33).

We report here a rapid method for releasing, subfractionating, and purifying ECP from *S. hyodysenteriae* (strain B204) in quantities which permit their biochemical characterization and molecular cloning as well as their evaluation in vaccination-challenge studies. We focus particular attention on a 38-kDa ECP. Cloning and DNA sequence analysis of the gene encoding this protein, which we designate *flaB1*, indicate that it is likely a component of the periplasmic flagella of *S. hyodysenteriae* B204.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. hyodysenteriae* B204 was obtained from Joann Kinyon, Iowa State University. Cells were grown anaerobically (80% nitrogen, 10% carbon dioxide, 10% hydrogen) from a 5 to 10% inoculum in a Braun Biostat M fermentor at 37°C and pH 6.8 in Difco brain heart infusion broth (37 g/liter) supplemented with 5% heat-inactivated fetal bovine serum, 0.5% glucose, and 20 mg of spectinomycin per liter.

Escherichia coli JM83 or W3110 was used for plasmid library construction, screening, and heterologous expression.

Cell fractionation and protein purification. Cells were harvested by centrifugation in late log phase (A_{600} = 1.5 to 2) 18 to 24 h after inoculation, with a yield of approximately 10 g (wet weight) per liter. Cell pellets were washed with 10 mM potassium acetate–150 mM potassium chloride (pH 4.75) and used immediately or frozen at −20°C.

ECP were released from the washed cell pellet by resuspension in 10 mM potassium acetate (pH 4.75) containing 0.2 to 10% Tween 20 at room temperature with gentle agitation for at least 10 min. Cell debris was removed by centrifugation at 10,000 × *g*. Sedimentable ECP were harvested from the supernatant by centrifugation at 100,000 × *g*. This pellet was solubilized in 25 mM Tris-Cl (pH 6.8)–6 M urea by sonication on ice until visually homogeneous with a Branson Cell Disruptor 200 (output, 7) followed by centrifugation at 100,000 × *g*. The urea-insoluble pellet was reextracted with 25 mM Tris-Cl (pH 6.8)–6 M urea and centrifuged at 100,000 × *g*.

Urea-solubilized ECP were filtered through a 0.45-μm-pore-size filter, acidified with trifluoroacetic acid (TFA) (0.1%), and loaded onto an analytical (4.6 by 250 mm) or preparative (21.5 by 250 mm) C_4 column (Vydac) which was equil-

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ibrated with 0.1% TFA. The columns were developed with a gradient of 0 to 100% acetonitrile-isopropanol (2:1)–0.1% TFA and monitored at 214 nm. Up to 500 ml of acidified, urea-solubilized ECP (50 to 150 mg of total protein) was routinely loaded on preparative columns, which were run at 10 ml/min. A gradient rate of 0.2%/min was used in the region where most of the ECP eluted (40 to 60%).

Cell fractions and purified proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the buffer system of Laemmli (19) on 10 or 12% acrylamide gels with molecular mass standards from Sigma (bovine serum albumin [66 kDa], ovalbumin [45 kDa], rabbit muscle glyceraldehyde 3-phosphate dehydrogenase [36 kDa], bovine erythrocyte carbonic anhydrase [29 kDa], bovine pancreatic trypsinogen [24 kDa], soybean trypsin inhibitor [20 kDa], bovine milk β-lactoglobulin [18 kDa], and bovine milk α-lactalbumin [14 kDa]). Gels were fixed and stained with Coomassie blue. Electrophoretic transfer of proteins from acrylamide gels to nitrocellulose membranes (PH79; Schleicher and Schuell) was performed as described by Towbin et al. (35). The membranes were probed overnight at 4°C with swine antiserum diluted (1:250) in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Antibody binding was visualized following a 2-h incubation with a peroxidase-conjugated rabbit anti-swine immunoglobulin G antibody in PBS–0.05% Tween 20 (United States Biochemical) and development with hydrogen peroxide and 4-chloro-1-napthol.

Peptide sequencing. Amino-terminal sequencing of proteins purified by reverse-phase high-pressure liquid chromatography (HPLC) was performed by sequential Edman degradation on an automated Applied Biosystems gas phase sequencer. Internal peptide sequences of purified proteins were determined after digestion with 1 μg of endoproteinase Lys-C (Boehringer Mannheim) per 75 μg of purified protein in 50 mM Tris-Cl (pH 8.5)–0.1% SDS for 4 to 12 h at 37°C and purification of peptide fragments on an analytical C_4 column that was developed with a gradient of 0 to 100% acetonitrile-isopropanol (2:1)–0.1% TFA and monitored at 214 nm.

Cloning and analysis of *S. hyodysenteriae* DNA. *S. hyodysenteriae* B204 DNA was prepared from a fresh 1-liter cell pellet of early-log-phase cells (optical density at 600 nm = 0.5) and purified in CsCl gradients as described by Maniatis et al. (23). Oligonucleotides were synthesized on a Biosearch 8700 automated DNA synthesizer and acrylamide gel purified prior to labeling with [γ-^{32}P]dATP (New England Nuclear) and T4 polynucleotide kinase (Boehringer Mannheim). Labeled oligonucleotides were used to probe Southern blots or to screen a pUC9 plasmid library (23). Genomic DNA was digested with *Hin*dIII and size fractionated on an agarose gel. The plasmid library was constructed from gel fractions which hybridized to selected oligonucleotide probes.

Manual DNA sequencing of both strands of plasmid DNA was performed by the dideoxy termination method of Sanger et al. (29). Deduced amino acid sequences were aligned with the GenBank (release 80.0), EMBL (release 36.0), SWISS-PROT (release 27.0), and PIR-Protein (release 38.0) data bases by using the FASTA algorithm of Pearson and Lippman (28).

A plasmid directing the heterologous expression of *S. hyodysenteriae flaB1* from pTrep2 (see Results) was constructed by an in-frame fusion at the *Nde*I site (FlaB1 amino acid 1) after filling in with T4 DNA polymerase (BRL) with the *Hin*cII site in pWHA93, a derivative of pWHA43 (24) which has a pUC polylinker inserted at the carboxy terminus of the calf prochymosin gene. The recombinant fusion protein was recovered from pWHA93-transformed *E. coli* W3110 as a Triton X-100-insoluble inclusion body according to the method of McCaman et al. (24).

Experimental challenge with *S. hyodysenteriae*. Yorkshire pigs approximately 8 weeks of age obtained from a disease-free herd in Ames, Iowa, were vaccinated intramuscularly with 2 ml of a 1:1 mixture of a mineral oil-paraffin adjuvant and PBS containing 100 μg of SDS-solubilized recombinant FlaB1 fusion protein. Placebo recipients were given injections of the adjuvant-PBS mixture without the fusion protein. Animals were boosted intramuscularly 5 weeks after priming.

Two weeks after being boosted, the animals were fasted for 24 h prior to the challenge, which was administered orally on two successive days via feed mixed with laboratory-grown *S. hyodysenteriae* B204. The challenge dose was estimated at 5×10^8 pathogens per animal per day on the basis of the microscopic titer of the laboratory culture. During the 6-week postchallenge period, animals were evaluated for clinical signs of swine dysentery. At the first appearance of blood in the stool, the presence of *S. hyodysenteriae* was confirmed by anaerobic culture of rectal swabs on spectinomycin-supplemented blood agar plates at 37°C.

Nucleotide sequence accession number. The DNA sequence shown in Fig. 4 has been submitted to the Genome Sequence Data Base and assigned accession number L34686.

RESULTS

Release of ECP with Tween 20 and characterization with SDS-PAGE. In our initial efforts to release and purify ECP from *S. hyodysenteriae*, we used low concentrations of SDS similar to those used by other investigators with *S. hyodysenteriae* and other spirochetes (7, 12, 15, 17, 25, 30). However, in our hands, SDS treatment frequently disrupted the cell wall and contaminated the ECP fraction with cytoplasmic proteins.

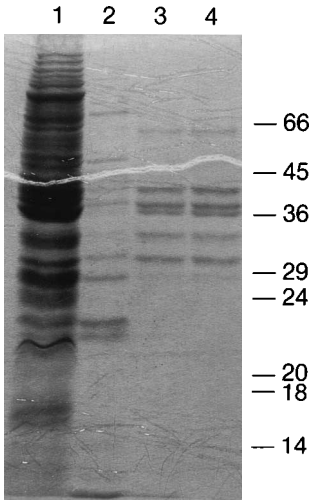

FIG. 1. SDS-PAGE of ECP released from *S. hyodysenteriae* (strain B204) by sequential extraction with increasing concentrations of Tween 20 in 10 mM potassium acetate, pH 4.75. Lanes: 1, whole-cell lysate; 2, 0.2% Tween 20; 3, 0.65% Tween 20; 4, 1.3% Tween 20. Molecular mass standards in kilodaltons are indicated.

We found that treatment of *S. hyodysenteriae* whole cells with 0.2% Tween 20 in 10 mM potassium acetate (pH 4.75) reproducibly released a subset of cellular proteins with molecular masses ranging from approximately 22 to 75 kDa without apparent cell lysis and release of cytoplasmic contaminants. Repeated extractions of the residual cell pellet with increasing concentrations of Tween 20 at pH 4.75 released additional quantities of ECP with a different SDS-PAGE profile but still without significant contamination by other cellular proteins (Fig. 1). We have been able to extract cells with up to 10% Tween 20 without cell lysis.

Six major proteins with molecular masses estimated at 42, 39, 38, 34, 31, and 29 kDa and two poorly resolved doublets with molecular masses estimated at 44/43.5 and 33.5/33 kDa could be recovered by ultracentrifugation of the proteins released by any of the Tween treatments (Fig. 2). The SDS-PAGE profile of the sedimented proteins is very similar to that of the proteins released by any of the sequential Tween treatments after the initial one. This suggests that the sedimentable proteins are components of detergent-stable macromolecular

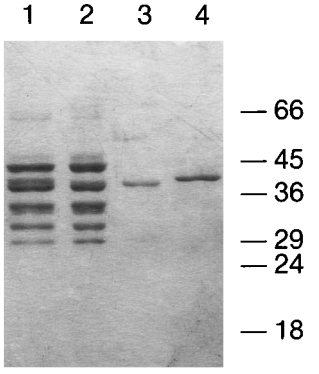

FIG. 2. SDS-PAGE of sedimentable ECP and solubilization with 6 M urea. Lanes: 1, sedimentable ECP; 2, urea-solubilized ECP, first extraction; 3, urea-solubilized ECP, second extraction; 4, urea-insoluble ECP. Molecular mass standards in kilodaltons are indicated.

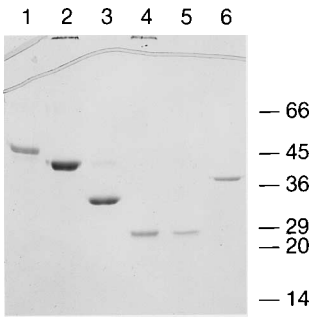

FIG. 3. SDS-PAGE of sedimentable ECP solubilized with 6 M urea and purified by reverse-phase HPLC. Lanes: 1, 43.5/44-kDa doublet; 2, 42-kDa ECP; 3, 34-kDa ECP; 4, 29-kDa ECP; 5, 29-kDa ECP; 6, 38-kDa ECP. Proteins in lanes 1 to 4 were purified from the first urea extraction of the sedimentable ECP. Proteins in lanes 5 and 6 were purified from the second urea extraction of the sedimentable ECP. Molecular mass standards in kilodaltons are indicated.

membrane and flagellar fragments while the proteins present primarily in the initial Tween supernatant represent soluble periplasmic proteins and solubilized extrinsic and intrinsic membrane proteins.

Purification of sedimented ECP by differential urea solubilization and reverse-phase HPLC. The sedimented proteins were further fractionated by two sequential extractions with 6 M urea. Seven of the 10 sedimented ECP (44, 43.5, 42, 34, 33.5, 33, and 31 kDa) were almost completely solubilized by the initial urea treatment (US1 fraction). All of the 39-kDa ECP and a portion of the 38- and 29-kDa ECP remained insoluble. The residual 38- and 29-kDa ECP were solubilized after a second urea extraction (US2 fraction). Only the 39-kDa ECP remained urea insoluble. No 39-kDa protein was evident in either of the urea-soluble fractions (Fig. 2).

The urea-solubilized proteins from the US1 and US2 cell fractions were further purified by reverse-phase HPLC. Column fractions greatly enriched for the 44/43.5-kDa doublet and the 42-, 34-, and 29-kDa ECP were obtained from US1. The 38- and 29-kDa ECP were recovered from the reverse-phase HPLC of the US2 fraction (Fig. 3).

Peptide sequencing of urea-solubilized ECP. Amino-terminal peptide sequencing (Table 1) was performed on the 44/43.5-kDa doublet and the 42- and 34-kDa ECP purified by reverse-phase HPLC from US1, on the 38- and 29-kDa ECP purified by reverse-phase HPLC from US2, on the urea-insoluble 39-kDa ECP, and on the 31-kDa ECP which had been purified by preparative SDS gel electrophoresis of US1. No amino-terminal sequence could be obtained from the 29- and 34-kDa ECP or the 44/43.5-kDa doublet, suggesting that these proteins have amino termini which are chemically blocked either in vivo or as a result of the purification method. Internal peptide sequences from reverse-phase HPLC-purified peptides were obtained for the 42-, 38-, 34-, 31-, and 29-kDa ECP after proteolytic digestion by endoproteinase Lys-C (Table 1).

Homology searches of protein sequence and translated nucleic acid data bases identified homologies between several of the peptides from the 38- and 31-kDa proteins and numerous flagellar proteins or gene products. A total of 191 amino acid residues from seven Lys-C fragments of the 42-kDa ECP were also determined (data not shown). All of the peptide sequences obtained from the internal peptides and 59 of the 61 residues determined from the amino terminus of the 42-kDa ECP are identical to the deduced amino acid sequence of the FlaA1 flagellar sheath protein of *S. hyodysenteriae* from isolate C5 (18). Homology searches using peptide sequences from the

TABLE 1. Amino-terminal and internal peptide sequences of *S. hyodysenteriae* B204 ECP

Protein mass (kDa)	N-terminal sequence	Internal peptide sequence
29	None detected	TDMYLESYREFDDPLDT, VEIFRDTLPAK, NITVQIPANIRQEEFRGQVEQGISFMGIHVKVDPRDSYGK, YYIYFDQLMAKTDMYLETYREEDDPLD
31	MIINNNISALNANRQLNLTGNSMTKTIAQLSXGMRINTAGDDAXGL	TIAQLSSGMRINTAGDDASGLAVSEK, MRSQYRGLQQATRNAQNGISFIQTTEG YLN, LNMLXGRFALSTXEN, SLMIATENTIASESVIRDADMASAMVAY XREQILQQ
34	None detected	YPNMRLFATKPYGMXNQXYN, SYNFXDLVPTVQK, AQTFDVXVXGGNYDYTMEMIFEDYRG YTYTLPLGSIRYIG, VVGEDGGNAGAATTEQPQEAQQXTEQ PQEAQ, GLRFMNRFRWSSPEERADNFVVLLDYF QTVV
38	MVINNNISAINAQRTLKFRQVDLKKDSAMISSGMRINQAGDDXXGLA	See Fig. 4
39	MYGDRDSWIDFLTHGNQFRARMDQLGFVLGNDXIKXTFXXR	
42	LXNSTLIDFALTGNADNLQAGEGDCNEVVPVAENLYNDNWVVWLN EDARLTENRRNSYVXNVD	See text
43.5/44	None detected	Not done

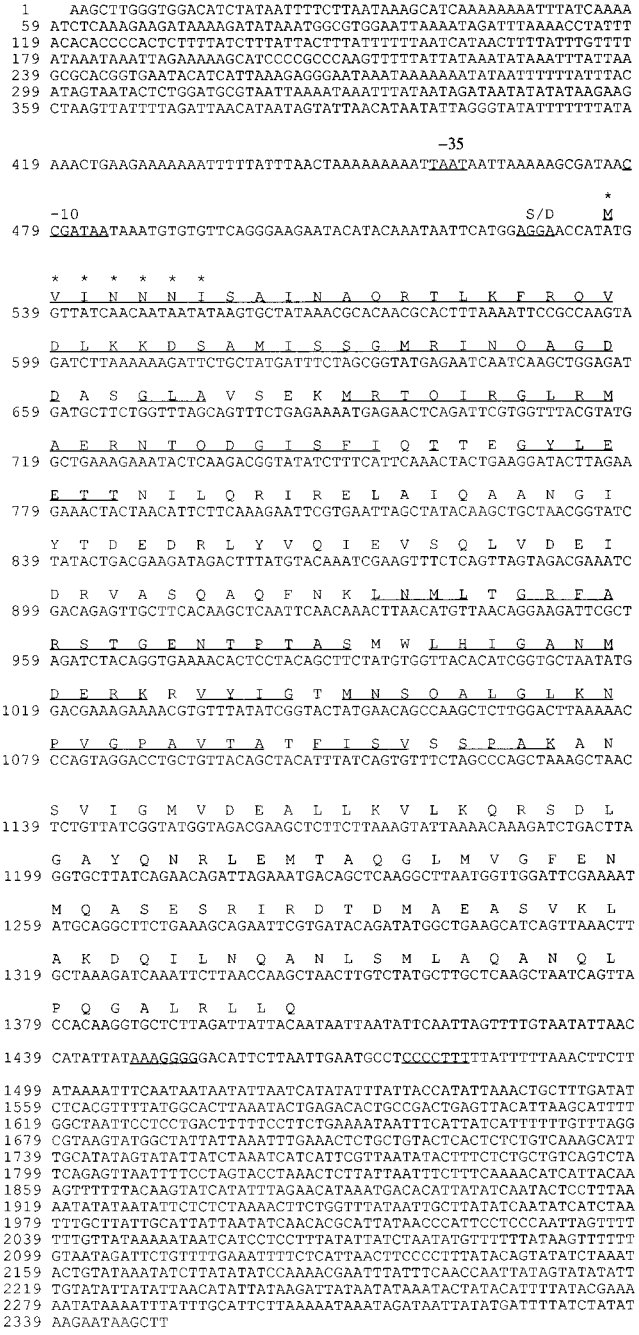

FIG. 4. Nucleotide sequence of a 2,350-bp *Hin*dIII fragment from *S. hyodysenteriae* (strain B204) and deduced amino acid sequence of the *flaB1* gene. Amino acids used to design COD 450 (GTN ATT/A/C AAT/C AAT/C AAT/C AT) are identified by an asterisk. Amino acids confirmed by peptide sequencing are underlined. A presumed ribosome binding site (S/D), −10 and −35 promoter sequences, and inverted GC-rich repeats downstream of the translational stop are also underlined.

39-, 34-, and 29-kDa ECP did not reveal strong homologies with any known or deduced protein sequence.

Molecular cloning and DNA sequence analysis of the *flaB1* gene encoding a flagellar core protein. The peptide sequence obtained from the amino terminus of the 38-kDa ECP was used to design a degenerate oligonucleotide probe (COD 450). This probe, which hybridized to 3.2- and 2.3-kbp fragments

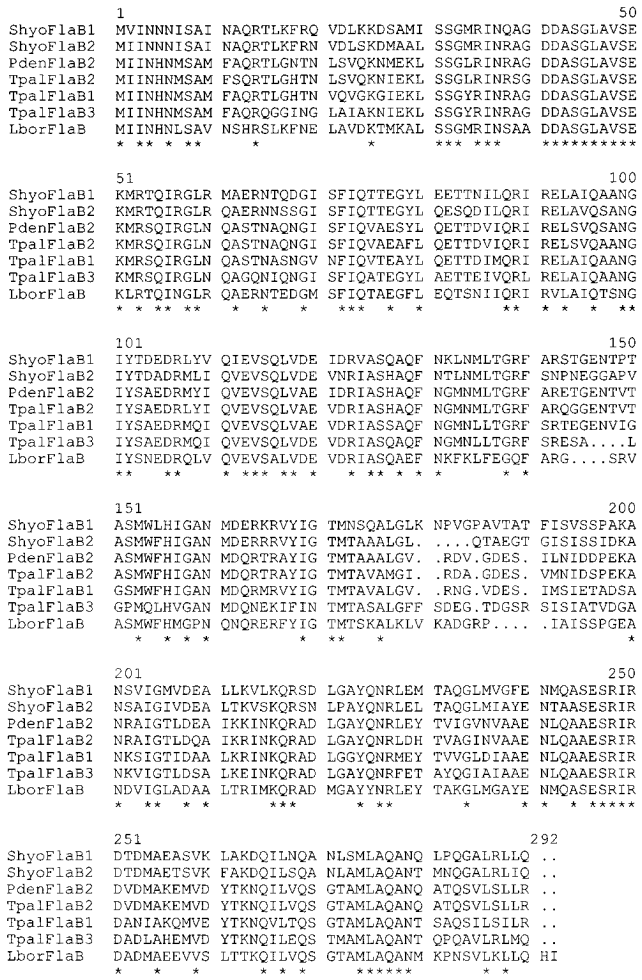

FIG. 5. Amino acid sequence alignment of FlaB proteins from *S. hyodysenteriae* (Shyo) (16), *T. phagedenis* (Pden) (22), *T. pallidum* (Tpal) (5, 27), and *L. borgpetersenii* (Lbor) (26). Amino acid residues conserved in all seven proteins are identified by an asterisk.

from a *Hin*dIII digest of genomic *S. hyodysenteriae* B204 DNA, was used to screen a plasmid library of size-fractionated, *Hin*dIII-digested B204 genomic DNA. One clone which was identified and sequenced, pTrep2, contained a 2,350-bp *Hin*dIII fragment with an 870-bp open reading frame encoding a protein with a calculated molecular mass of 32 kDa (Fig. 4). The first 41 amino acids of the deduced protein sequence are identical to the amino-terminal sequence obtained from the 38-kDa protein. In addition, the nucleotide sequence predicts the presence of three additional internal peptides which match the peptide sequence obtained from endoproteinase Lys-C fragments of the 38-kDa protein (Fig. 4).

The predicted full-length protein sequence is homologous to those of numerous bacterial flagellar proteins, particularly the FlaB2 protein from *S. hyodysenteriae* (74%) (16), as well as the FlaB proteins from the spirochetes *Treponema phagedenis* (61%), *Leptospira borgpetersenii* (60%), and *Treponema pallidum* (60, 58, and 57%) (5, 22, 26, 27). One hundred seven of the amino acid residues (37%) are conserved in all seven of the FlaB flagellar core proteins from these spirochetes (Fig. 5). Accordingly, we designate the *S. hyodysenteriae* flagellar gene cloned in pTrep2 *flaB1* and its encoded protein FlaB1.

The translated *flaB1* sequence and the amino-terminal pep-

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-35                    -10
TTAATAATTAAAAAGCGATAACCGATAA  S. hyodysenteriae flaB1
CTAAA       N16        CCGATAT  σD consensus
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FIG. 6. Comparison of nucleotide sequences upstream of the *S. hyodysenteriae flaB1* initiation codon with consensus σ^D promoter sequences from *Bacillus subtilis* (10). Presumptive −10 and −35 sequences are underlined. A duplicated sequence homologous to the −10 sequence is in boldface.

tide sequence of purified FlaB1 from *S. hyodysenteriae* indicate that this protein, like most other flagellar proteins, lacks a signal peptide directing transmembrane transport and contains no cysteines. A consensus Shine-Dalgarno sequence is located 9 nucleotides upstream of the presumptive ATG initiation codon, which corresponds to the amino terminal sequence determined for FlaB1. There is a potential in-frame initiation codon 48 nucleotides upstream of the presumptive initiation codon. However, this potential initiation codon is not preceded by a consensus Shine-Dalgarno sequence, and initiation of translation at this location would not generate a sequence with the characteristics of a typical signal peptide (36).

At 52 and 59 bp upstream of the translational initiation codon of *flaB1* in *S. hyodysenteriae*, there are repeated sequences with strong homology to the −10 sequences found upstream of the presumptive transcriptional start sites of flagellar genes and genes subject to catabolite repression which are regulated by the alternative sigma factor, σ^D (σ^{28}) (Fig. 4 and 6) (10). A sequence similar to the consensus −35 σ^D sequences can be found 16 bp upstream of the most 3′ presumptive −10 sequence. Similar presumptive regulatory sequences have been identified upstream of the *flaB* genes from *T. pallidum* and *T. phagedenis* (5, 22, 27) but have not been identified preceding the *flaA1* and *flaB2* flagellar genes from *S. hyodysenteriae* (16, 18).

G-rich and C-rich inverted repeats are located downstream of the predicted *flaB1* translational stop and may play a role in the rho-independent transcription termination of *flaB1* (Fig. 4).

The *flaB1* gene in *S. hyodysenteriae* does not appear to be part of a flagellar operon, in contrast to the *flaB1* and *flaB3* genes from *T. pallidum* (5). The *Hin*dIII fragment cloned in pTrep2 contains more than 400 nucleotides preceding *flaB1* and more than 900 nucleotides following the gene. No potential open reading frame which would encode a protein with homology to FlaB1 or other reported flagellar proteins, including FlaB2 from *S. hyodysenteriae* (16), could be identified in these regions.

Heterologous expression of *flaB1* in *E. coli* and experimental challenge of animals vaccinated with recombinant FlaB1. We could not detect heterologous expression of *flaB1* visually or immunologically in pTrep2-transformed *E. coli* grown in either L broth or M9 salts with glycerol. Expression in *E. coli* was accomplished by fusing the *flaB1* open reading frame from pTrep2, beginning at the *Nde*I site encoding amino acid 1, to the 3′ end of the gene encoding the first 366 amino acids of calf prochymosin in the expression vector pWHA93. The resulting plasmid, pTrep9, encoded a 72-kDa fusion peptide which was recovered as an insoluble inclusion body from lysed cells (Fig. 7A). Serum antibody from pigs immunized with the SDS-solubilized purified fusion protein recognized the FlaB1 protein, as well as the homologous FlaB2 protein, in Western blots (immunoblots) of sedimentable ECP from *S. hyodysenteriae* (Fig. 7B). All five of the animals vaccinated with the fusion peptide and the five placebo-vaccinated controls were susceptible to an experimental challenge with an inoculum of *S. hyodysenteriae* B204. A slight delay in the mean onset of dysentery in the group vaccinated with recombinant FlaB1 (24.0 ± 10.7 days) compared with that in the placebo group (14.4 ± 4.7 days) has not been reproduced in other studies with this antigen. We have also been unable to demonstrate a reduction in either the incidence or severity of experimentally induced dysentery in swine after vaccination with FlaB1 purified from *S. hyodysenteriae* (data not shown).

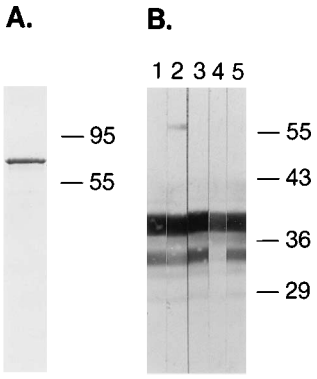

FIG. 7. (A) SDS-PAGE of purified recombinant FlaB1-prochymosin fusion protein. (B) Western blot of *S. hyodysenteriae* sedimentable ECP probed with sera (1:250) from five swine vaccinated with purified recombinant FlaB1-prochymosin fusion protein. Prevaccination serum contained no detectable anti-ECP antibodies. Molecular mass standards in kilodaltons are indicated.

DISCUSSION

We have used the nonionic detergent Tween 20 at pH 4.75 to disrupt the outer membrane and gently release ECP from intact *S. hyodysenteriae* (strain B204). Under these conditions the bacterial cell wall apparently remains intact and permits the reextraction of ECP with increasing concentrations of detergent. We have found that Tween concentrations of up to 10% do not appear to disrupt the integrity of the cell wall, and we have been able to routinely recover 50 mg of ECP per 10 g of cell paste.

Our method and those employed by several other investigators (11, 15, 17, 21, 25, 30, 32, 34, 37) all share a strategy of disruption of the microbe by mechanical or chemical means followed by differential centrifugation to remove contaminating proteins. As has been the case with these other studies, we have focused our attention on proteins which could be recovered and concentrated by virtue of their sedimentation during high-speed centrifugation. We note, however, that the particular method employed in this study also permits the isolation of soluble periplasmic proteins as well as detergent-solubilized intrinsic and extrinsic membrane proteins which may have been discarded by other techniques and may be of immunological or physiological interest.

The Coomassie blue-stained SDS-PAGE profiles of the sedimentable ECP obtained by all of these methods are similar. Nevertheless, the sedimentable fractions generated by these different published methods are not identical in content. Each has its own distinctive characteristics which may be attributable to differences in bacterial strains and cultural conditions as well as to the differences in the specific methods used to isolate these fractions. Ongoing analysis of the common and distinctive antigens contained within these various preparations will contribute to a more complete understanding of the molecular topography of *S. hyodysenteriae* and of their immunological relevance to the infected or vaccinated host.

This study also presents a method for differential urea sol-

ubilization and chromatographic purification of several of the individual proteins in the sedimentable ECP. The quantities of proteins isolated enable further biochemical, immunological, and molecular biological characterization of some of the major and minor constituents of the ECP. The amino-terminal and internal peptide sequence data presented in this study for six of these proteins indicate that three of them, the 39-, 34-, and 29-kDa ECP, have not been previously characterized. The 34-kDa ECP has an apparent molecular mass similar to that reported for FlaB2 from *S. hyodysenteriae* (16). However, the peptide sequence obtained for the 34-kDa ECP indicates that it is not homologous to FlaB2. Since antiserum raised against the recombinant FlaB1 protein cross-reacts with a 34-kDa ECP, it is likely that the ECP preparation contains two distinct proteins of similar mass, only one of which was subsequently purified and partially characterized in this study. We note that a 34-kDa protein which may correspond to FlaB2 elutes as a part of a poorly resolved group of peaks near the end of the reverse-phase HPLC separation of the US1 fraction. Three other proteins, the 42-, 38-, and 31-kDa ECP, correspond to flagellar proteins FlaA1, FlaB1, and FlaB3, respectively, which have been partially characterized by other investigators (17). The *flaA* gene from *S. hyodysenteriae* isolate C5 has been cloned and sequenced (18) and encodes a flagellar sheath protein with a sequence which is nearly identical to the protein sequence obtained for the 42-kDa protein we have isolated from strain B204, as well as to the predicted peptide sequence encoded by the *flaA* gene which we have cloned from strain B204 (data not shown).

The 38-kDa ECP was our initial target for more-detailed characterization and cloning because it was strongly reactive with antisera from convalescent swine (data not shown) and was obtained easily as the major component in the US2 cell fraction. DNA sequence analysis of the gene encoding this protein, which we designate *flaB1*, identifies an 870-bp open reading frame encoding a 32-kDa protein with a sequence which is very homologous to the predicted amino acid sequence of the FlaB2 flagellar core protein from *S. hyodysenteriae* and to those of the FlaB flagellar proteins from several other spirochetes. The disparity between the predicted (32-kDa) and observed (38-kDa) molecular masses for FlaB1 is noteworthy. Smaller differences of 2 to 3 kDa between the predicted and observed molecular masses of other *flaB* gene products have been reported by other investigators and are generally attributed to anomalous migration of flagellar proteins on SDS-PAGE (5, 16, 22, 26, 27). The comparatively large difference seen with FlaB1 from *S. hyodysenteriae* may be indicative of posttranslational modification of flagellar proteins in *S. hyodysenteriae*. Posttranslational modification has been considered as a potential explanation for a similarly large disparity of approximately 10 kDa between the predicted and observed masses of FlaA1 from *S. hyodysenteriae* (18). Glycoprotein staining of FlaA1 and modification of its electrophoretic mobility after enzymatic treatment with N-glycosidase F have recently been reported (20). We note that peptide sequencing of several of the internal Lys-C fragments of FlaB1 failed to detect threonine residues predicted by the nucleotide sequence at amino acids 137 and 171 even though other threonine residues in these peptides where identified. The inability to detect these residues suggests that the mature peptide may be posttranslationally modified at these sites, perhaps by O-linked sugar additions, which would consequently increase the apparent molecular weight as determined by SDS-PAGE.

An alternative explanation for the comparatively large difference seen between the observed and predicted molecular masses of FlaB1 can be found in the multiplicity of related flagellar genes and proteins in *S. hyodysenteriae*. Three different FlaB proteins from *S. hyodysenteriae* have been partially characterized by ourselves and others (17), and the existence of either three or four *flaB* genes has been inferred from Southern blots (16, 38). It is clear from the distinctive amino acid sequences of the characterized FlaB proteins that *flaB1* can encode only the 38-kDa flagellar protein. However, it is possible that *flaB1* encodes an uncharacterized FlaB protein and that the 38-kDa ECP is encoded by a different *flaB* gene. Although this possibility cannot be completely discounted, we believe that it is unlikely because of the complete agreement between the nucleotide sequence of *flaB1* and the 133 amino acid residues determined for the 38-kDa ECP.

The nucleotide sequence homology upstream of the *flaB1* gene with the −10 and −35 sequences upstream of other flagellar genes and σ^D catabolite-repressible promoters suggests that the synthesis of flagellar proteins in *S. hyodysenteriae*, as is the case with other bacteria, is part of a transcriptionally regulated cascade involving proteins responsible for motility and chemotaxis which responds to the environmental conditions encountered by the pathogen (6, 9). The observation that motility and chemotaxis can be observed with *S. hyodysenteriae* isolated from infected tissues but not with pathogens cultured in vitro (14) suggests that the synthesis and assembly of a completely functional system of sensory and motility organelles could depend upon environmental cues which may not be duplicated in in vitro cultures.

It has been proposed that the capacity for motility and chemotaxis is a crucial factor in the colonization of swine by *S. hyodysenteriae* (14). An immune response which could interfere with the spirochete's motility and ability to colonize the colon is an appealing vaccine target. However, we have been unable to demonstrate a reduction in either the incidence or severity of dysentery in swine vaccination-challenge studies after immunization with either authentic or recombinant *S. hyodysenteriae* FlaB1. An immune response directed solely at the FlaB1 core protein of the flagella may be ineffective because of the barriers afforded by peripheral flagellar proteins and the pathogen's outer membrane. In *T. pallidum*, flagellar core proteins in isolated flagella are accessible to antibodies (1), suggesting that an immune response to a core flagellar protein may still be effective if the outer membrane can be breached. In contrast to the results presented in this study, it has been reported that mice vaccinated with a cloned *S. hyodysenteriae* endoflagellar antigen expressed in *E. coli* were protected from an oral challenge of the pathogen (4). Those investigators also reported that serum taken from the vaccinated animals prior to challenge was bactericidal in a complement-dependent in vitro assay and speculated that the outer membrane does not afford a complete barrier to antiflagellar antibodies in vivo. Similar in vitro sensitivity of *T. pallidum* to antiflagellar antibodies has been reported (2) and was subsequently associated with an immune response which must also disrupt the outer membrane (3). Western blots using sera from animals vaccinated with FlaB1 in this study demonstrate that there were high levels of circulating antibody against the vaccinal antigen but do not indicate whether a comparable immune response was present at the site of infection or whether the FlaB1 antigens were accessible to it. While our data indicate that a serological response to FlaB1 alone is not sufficient to confer protection against an experimental challenge, they cannot determine whether an immunological response, serological or otherwise, to one or more flagellar proteins of *S. hyodysenteriae* is an essential component of the protection acquired by convalescent pigs as a result of infection.

ACKNOWLEDGMENTS

We thank Ray Muffly for oligonucleotide synthesis, P. Schaefer Price for plasmid library construction and screening, Daniel Wong and John McClary for DNA sequencing, and Michael Kennedy for reviewing the manuscript.

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