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Cloning and DNA Sequence Analysis of an Immunogenic Glucose-Galactose MglB Lipoprotein Homologue from *Brachyspira pilosicoli*, the Agent of Colonic Spirochetosis†

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Colonic spirochetosis (CS) is a newly emerging infectious disease of humans and animals caused by the pathogenic spirochete *Brachyspira* (formerly *Serpulina*) *pilosicoli*. The purpose of this study was to characterize an antigen that was recognized by antibodies present in sera of challenge-exposed pigs. The gene encoding the antigen was identified by screening a plasmid library of human *B. pilosicoli* strain SP16 (ATCC 49776) genomic DNA with hyperimmune and convalescent swine sera. The predicted amino acid sequence encoded by the cloned *B. pilosicoli* gene had a high degree of similarity and identity to glucose-galactose MglB lipoprotein. Located 106 bp downstream of the putative *mglB* gene was a 3′-truncated open reading frame with 73.8% similarity and 66.3% identity to *mglA* of *Escherichia coli*, suggesting a gene arrangement within an operon which is similar to those of other bacteria. A single copy of the gene was present in *B. pilosicoli*, and homologous sequences were widely conserved among porcine intestinal spirochetes *Serpulina intermedia*, *Brachyspira innocens*, *Brachyspira murdochii*, and the avian *Brachyspira alvinipulli*, but not in porcine *Brachyspira hyodysenteriae*, human *Brachyspira aalborgi*, and porcine *Treponema succinifaciens*. The deduced molecular weight of the mature MglB lipoprotein was consistent with expression by the cloned gene of a polypeptide with an apparent molecular weight of 36,000, as determined by Western blot analysis and [^{3}H]palmitate labeling. Because mucin is the principal constituent of the colonic mucus gel and consists of glycoproteins that can serve as the substrate for growth and chemotaxis of *B. pilosicoli* in vitro, a role for MglB in mucosal localization of the spirochete appears consistent with the pathogenesis of CS. However, the presence of homologous sequences in closely related but nonpathogenic commensal spirochetes suggests that other virulence determinants may be required for pathogenesis.

Colonic spirochetosis (CS) is a newly emerging infectious disease of humans and animals caused by the pathogenic spirochete *Brachyspira* (formerly *Serpulina*) *pilosicoli* (6, 7, 9, 38, 56, 58). Infection with *B. pilosicoli* or lesions consistent with CS have been recorded in a wide variety of hosts including human beings (6, 22, 56, 61, 62), nonhuman primates (8, 9, 36), pigs (2, 6, 7, 11, 13, 27, 52, 54, 55), dogs (6, 8, 10), commercial chickens, and various species of wild and zoo birds (39). The prevalence of CS among adults in the United States and Europe ranges between 4.5 and 32.2% (45). By contrast, infection with *B. pilosicoli* is endemic among villagers in Papua New Guinea; 93.6% of the population is infected for a calculated average duration of about 4 months (61). Porcine, canine, human, and monkey strains of *B. pilosicoli* are closely related and cause CS in chick and swine infection models (6, 9, 10, 11, 15, 35, 36, 54, 57–60). Similarly, laboratory mice can be colonized for up to 30 days with human, porcine, and avian *B. pilosicoli* strains (46). Collectively, these data suggest that *B. pilosicoli* may be zoonotic, and this has public health significance.

By analogy with the pathogenic intestinal spirochete of swine *Brachyspira hyodysenteriae*, initial colonization of the colon by *B. pilosicoli* appears to involve motility-regulated mucin association (24, 33, 64). Multiplication of the spirochetes in close proximity with the mucosal surface and inside the lumina of the crypts (54) is followed by intimate attachment along the apical membrane of enterocytes causing effacement of microvilli (7, 8, 11, 37, 52, 56, 57, 59). Comparative studies with cultured enterocytes and infection models suggest a specific spirochete ligand-host cell membrane receptor interaction during intimate attachment of *B. pilosicoli* (35, 36). Penetration of the colonic epithelium and invasion of *B. pilosicoli* into the submucosal connective tissue are seen in humans, pigs, and dogs with naturally occurring disease and in experimentally infected pigs and chicks (8, 35). Translocation of *B. pilosicoli* to extraintestinal sites including the bloodstreams of terminally ill human patients also has been documented (62). Because *B. pilosicoli* is emerging as a human pathogen and because these organisms have not been studied in detail, there is a need to more fully understand basic mechanisms involved in intestinal colonization and disease.

Considerable genetic information for the pathogenic spirochetes *Borrelia burgdorferi*, the cause of Lyme disease (16), and *Treponema pallidum*, the syphilis spirochete (17), is available. With the exception of the role of NADH oxidase expression in colonization of the swine colon by *B. hyodysenteriae* (51) and of flagellar gene expression in the motility of *B. hyodysenteriae* (25, 44) and the oral spirochete *Treponema denticola* (28), little is known about the structure-function relationship of specific gene products in the pathogenesis of spirochetal diseases.

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TABLE 1. Sources, origins, and presence of *B. pilosicoli mglB* gene homologue among intestinal spirochetes

Species and strain	Origin (host/country[c])	*mglB* gene[a]	Source[b]
B. hyodysenteriae			
B78 (ATCC 27164)	Pig/U.S.	−	1
B204	Pig/U.S.	−	1
S. intermedia			
PWS/A (ATCC 51140)	Pig/U.K.	−	2
889	Pig/Australia	+	3
AN26:93	Pig/Sweden	+	4
B. innocens			
B256 (ATCC 29786)	Pig/U.S.	−	5
C301	Pig/Sweden	+	4
C378	Pig/Sweden	+	4
4/71	Pig/U.K.	+	2
B. murdochii			
56-150 (ATCC 51254)	Pig/Canada	+	6
155-20	Pig/Australia	+	3
B. pilosicoli			
P43/6/78 (ATCC 51139)	Pig/U.K.	+	2
UNL-8	Pig/U.S.	+	7
SP16 (ATCC 49776)	Human/U.S.	+	8
B. aalborgi			
513 (ATCC 43994)	Human/Denmark	−	3
B. alvinipulli			
C1 (ATCC 51933)	Chicken/U.S.	+	5
T. succinifaciens			
6091 (ATCC 33096)	Pig/U.S.	−	2

[a] As determined by subjecting *Hae*III-digested chromosomal DNA of each strain to Southern blot hybridization with a DIG-labeled ORF-2 probe prepared by PCR amplification of pPZD1003-36 with primers PZ3-F and PZ3-R. +, presence of homologue; −, absence of homologue.

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[c] U.S., United States; U.K., United Kingdom.

We have shown that swine that recovered from CS develop serum immunoglobulin G (IgG) antibodies to several *B. pilosicoli* antigens (65; P. Zhang, X. Cheng, M. Mathiesen, and G. E. Duhamel, Abstr. 79th Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. 54, 1998). The purpose of this study was to characterize an antigen recognized by antibodies present in sera obtained from pigs that recovered from CS. The present communication describes the cloning, sequencing, and expression of a *mglB* gene homologue (encoding a protein formerly known as β-methylgalactoside) in *B. pilosicoli*.

(This report represents a portion of a thesis submitted by P. Zhang to the University of Nebraska in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Bacterial strains and growth conditions. The reference and field isolates of intestinal spirochetes used in this study are presented in Table 1. The spirochetes were propagated in prereduced anaerobically sterilized Trypticase soy broth as described previously (12). Broth cultures were grown to late logarithmic phase (approximately 3 days; 10^8 cells per ml) while being stirred constantly at 37°C under a 10% hydrogen–10% carbon dioxide–80% nitrogen atmosphere. *Escherichia coli* strain DH5α (GIBCO-BRL, Gaithersburg, Md.) was grown in Luria-Bertani (LB) broth or LB agar at 37°C. For library screening, recombinant *E. coli* cells were grown on LB agar containing 100 μg of ampicillin, 12 μg of isopropyl-β-D-thiogalactopyranoside (IPTG), and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml at 37°C.

Production of convalescent and hyperimmune sera. Sera were obtained from conventional weaned pigs prior to inoculation and on day 49 after oral inoculation with either sterile medium or *B. pilosicoli* strain UNL-8 as described previously (11, 65). Equal volumes of sera collected from two pigs were pooled together. Pigs were considered convalescent when they had developed diarrhea and shed *B. pilosicoli* in their feces within the first weeks postinoculation (p.i.) but had become culture negative and had shown no colonic lesions at necropsy on day 49 p.i. Hyperimmune sera were produced by parenteral immunization of two 5-week-old conventional pigs with O_2-killed *B. pilosicoli* strain UNL-8 mixed in Freund's incomplete adjuvant. Briefly, a broth culture was bubbled with O_2 for 6 h at 4°C and washed three times with phosphate-buffered saline (PBS; pH 7.2). A volume of 200 μl containing 10^{10} spirochetes/ml in PBS was mixed with 800 μl of Freund's incomplete adjuvant and administered intramuscularly and subcutaneously on days 1, 14, 28, 35, and 49. On day 63, serum was harvested from each pig and pooled. The serum IgG antibody titers of hyperimmune and convalescent sera were estimated using a *B. pilosicoli* whole-cell enzyme-linked immunosorbent assay as described previously (L. N. Fisher, G. E. Duhamel, M. R. Mathiesen, and R. J. Bernard, Abstr. 71st Annu. Meet. Conf. Res. Workers Anim. Dis., abstr. 70, 1990). Prior to library screening and Western blot analysis, the sera obtained from control and convalescent swine and hyperimmune swine were absorbed with *E. coli* DH5α transformed with either cloning vector pBluescript II KS(+) (pBSK$^+$) (Stratagene Cloning Systems, La Jolla, Calif.) or pCR2.1 (Invitrogen, Carlsbad, Calif.). Briefly, *E. coli* cells grown to a density of 10^9 per ml were harvested by centrifugation (10,000 × *g*; 10 min) and resuspended to 0.02 of the original volume in PBS. Half of the cells were lysed by 10 10-s cycles of sonication (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) interspersed with cooling on ice, whereas the other half of the cells were boiled for 15 min. Then, 1/2 volume of sonicated and 1/2 volume of boiled *E. coli* cells were mixed together and incubated with serum for 16 h at 4°C. At the end of the incubation period, the serum was centrifuged (10,000 × *g*; 15 min) to remove precipitates and kept at −20°C until needed.

Isolation of chromosomal DNA. Chromosomal DNA of *B. pilosicoli* was purified as described previously (15), except that the cells were lysed by the addition of 0.05% (wt/vol) sodium dodecyl sulfate (SDS; Sigma, St. Louis, Mo.) and 1 mg of proteinase K (GIBCO-BRL)/ml. The concentration of DNA resuspended in sterile H_2O was estimated by fluorometry against a *Clostridium perfringens* DNA standard (DyNA Quant 200 fluorometer; Hoefer Pharmacia Biotech Inc., San Francisco, Calif.).

Library construction and screening. A genomic library of *B. pilosicoli* strain SP16 was prepared by complete digestion of purified chromosomal DNA with restriction enzyme *Hin*dIII (GIBCO-BRL), ligation into phagemid vector pBSK$^+$, transformation of competent *E. coli* DH5α by electroporation at 2,500 V and 25 μF (Gene Pulser; Bio-Rad, Hercules, Calif.), and plating onto LB agar containing ampicillin, IPTG, and X-Gal. The plasmid library was screened by colony blotting with absorbed hyperimmune (1:250 dilution) swine serum by a modification of a previously described method (12). Briefly, after lysis in a chloroform vapor chamber, replica membranes (Hybond-N; Amersham Life Science, Piscataway, N.J.) were incubated overnight in lysis-blocking solution (50 mM Tris-base [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 5% nonfat dried milk, and 2 μg of DNase I, 40 μg of lysozyme, and 100 μg of chloramphenicol/ml) at room temperature. The membranes were incubated at room temperature with rocking sequentially with serum for 2 h, followed by biotin-labeled goat anti-swine IgG (heavy and light chains) antibody (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) for 1 h, peroxidase-labeled streptavidin (Kirkegaard & Perry) for 45 min, and 4-chloro-1-naphthol (Kirkegaard & Perry) for 10 min. Three 5-min washes with buffer (5% nonfat dry milk and 0.05% Nonidet P-40 in 50 mM Tris-base [pH 7.5], 150 mM NaCl) were performed between each incubation step. Immunopositive clones were identified on the basis of development of a dark-purple precipitate.

DNA sequencing and analysis. Serial 5′ unidirectional 300- to 400-bp deletions of the DNA insert of an immunopositive clone were generated by sequential digestion with exonuclease III as recommended by the manufacturer (double-stranded nested deletion kit; Pharmacia Biotech, Piscataway, N.J.). The DNA of selected clones obtained from the deletion library was sequenced (Sequencing Facility, Iowa State University, Ames), and nucleotide and deduced amino acid sequences were analyzed using Genetics Computer Group software, version 8.1 (University of Wisconsin Biotechnology Center, Madison), PSORT, and ExPASy Proteomics and compared with available sequences in the GenBank, EMBL, DDBJ, and PDB databases using the BLAST program. The complete nucleotide sequence of 3′-end-truncated open reading frame 2 (ORF-2) was obtained by PCR amplification using synthesized (Integrated DNA Technologies, Inc., Coralville, Iowa) oligonucleotide primers PZ1-F, designed on the basis of DNA sequence analysis of ORF-2 (Table 2 and Fig. 1), and PZ1-R, a degenerate oligonucleotide corresponding to a region carrying highly conserved domains of the *E. coli*, *Salmonella enterica* serovar Typhimurium, and *T. pallidum mglA* genes located downstream of *mglB* (Table 2 and Fig. 1). The primers were used for amplification (GeneAmp PCR system 9600; Perkin-Elmer Corp., Norwalk,

TABLE 2. Oligonucleotide primers used for amplification and sequencing of *B. pilosicoli mglB*

Primer	Sequence (5′–3′)	*B. pilosicoli* or *E. coli* positions[a]
PZ1-F	GGCACTACTTGGACTTTAGATG	1392–1413
PZ1-R	TTNAA(G)T(C)TCT(C)TGA(G)TGNACCAT	1714–1695*
PZ2-F	ACACATTAAGCCCTGCAC	60–77
PZ2-R	GATATGCCCTTCATCTCAAG	1637–1618
PZ3-F	TTACAGTATCCTGCGGCG	490–507
PZ3-R	GGATCTTTACCAGCAGCAAC	1384–1365

[a] *, *E. coli* positions; GenBank accession no. gi|146852|gb59444.1.

Conn.) of purified chromosomal DNA from *B. pilosicoli* strain SP16 in a total volume of 75 μl containing 4 mM $MgCl_2$; 1× PCR buffer; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP; a 1-μM concentration of each primer; and 1.5 U of *Taq* DNA polymerase (GIBCO-BRL) in sterile filtered autoclaved water. Initial denaturing was for 5 min at 94°C and was followed by 30 cycles (45 s at 94°C, 45 s at 50°C, and 60 s at 72°C). For sequencing, PCR products were ligated with pCR2.1 (Invitrogen) and transformed into *E. coli* DH5α. Full-length ORF-2 was amplified by following the manufacturer's recommended PCR procedure (Expand high-fidelity PCR system; Boehringer Mannheim, Indianapolis, Ind.), which involved using oligonucleotide primers PZ2-F, corresponding to a sequence located upstream of ORF-2, and PZ2-R, corresponding to a sequence located immediately downstream of ORF-2 (Table 2, Fig. 1). The resulting amplified ORF-2 products were ligated with vector pCR2.1 (Invitrogen) to create pPZD1003-36 (Fig. 1) and transformed into *E. coli* DH5α.

SDS-PAGE and Western blotting. Log-phase broth cultures of *B. pilosicoli* or recombinant *E. coli* were centrifuged (12,000 × *g*; 30 s), and the cells were washed with ice-cold 0.05 M Tris-HCl (pH 7.4). The cell pellet was resuspended in sterile distilled H_2O, mixed with an equal volume of 2× sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue), boiled for 10 min, separated by SDS–10% polyacrylamide gel electrophoresis (SDS–10% PAGE), and electrotransferred to nitrocellulose (0.2-μm pore size; Midwest Scientific, Valley Park, Mich.) as previously described (14). After being blocked, the membranes were reacted with absorbed sera obtained from either control (1:100 dilution), convalescent (1:100 dilution), or hyperimmune (1:250 dilution) swine and diluted in buffer (5% nonfat dry milk and 0.05% Nonidet P-40 in 50 mM Tris-base [pH 7.5]–150 mM NaCl) and developed exactly as described above for colony blots of the plasmid library. Immunopositive bands were identified on the basis of development of a dark-purple precipitate.

Labeling of lipoproteins with [^{3}H]palmitate. *E. coli* strain DH5α cells transformed with pPZD1003-36 or control pCR2.1 and grown in LB medium containing 100 μg of ampicillin/ml to an optical density at 600 nm of 0.5 were mixed with [^{3}H]palmitic acid (Amersham, Arlington Heights, Ill.) to a final concentration of 25 μCi/ml and incubated for 3 h at 37°C as previously described (53). After the cells were washed three times with PBS, the radiolabeled lipoprotein bands were visualized by autoradiography after separation by SDS–12.5% PAGE.

Determination of gene copy number and homologous sequences among intestinal spirochetes by Southern blotting. The number of copies of the gene containing ORF-2 was determined by digestion of *B. pilosicoli* strain SP16 chromosomal DNA with restriction enzymes *Hin*dIII, *Xba*I, *Nsi*I/*Sca*I, *Eco*N1/*Sca*I, and *Eco*N1/*Spe*I and hybridization with a digoxigenin (DIG)-labeled probe prepared by PCR amplification of pPZD1003-36 with oligonucleotide primers PZ3-F and PZ3-R (Table 2, Fig. 1) as previously described (15). Briefly, purified pPZD1003-36 DNA was reacted with an 0.75 μM concentration of each primer in a total volume of 50 μl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 4 mM $MgCl_2$; 0.2 mM (each) dATP, dCTP, dGTP, and dTTP (GIBCO-BRL); 0.02 mM DIG-11-dUTP (Boehringer Mannheim), and 2.5 U of *Taq* polymerase (GIBCO-BRL), and the mixture was subjected to 35 cycles of amplification (94°C for 40 s, 58°C for 40 s, and 72°C for 60 s). The presence of sequences homologous to ORF-2 among intestinal spirochetes (Table 1) was determined by Southern blot hybridization of purified chromosomal DNA digested with restriction endonuclease *Hae*III with the DIG-labeled ORF-2 probe as previously described (15) except that prehybridization, hybridization, and the posthybridization washes were done at 68°C.

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to GenBank and has been assigned accession no. AF200741.

RESULTS

Identification of pPZD1003. The IgG antibody titers of convalescent and hyperimmune swine sera were 400 and 6,400, respectively. Nine immunopositive clones, designated pPZD1001 through pPZD1009, were identified by screening approximately 10,000 recombinant clones of *B. pilosicoli* by colony blotting with absorbed hyperimmune swine serum. Because clones pPZD1003 and pPZD1004 showed strong immunoreactive bands when reacted with convalescent serum, they were selected for further studies. Clones pPZD1001 and pPZD1002 expressed proteins that were recognized only by hyperimmune serum, whereas hyperimmune and convalescent sera did not react with clones pPZD1005 through pPZD1009, as shown by immunoblot analysis. Southern blot cross-hybridization of clones pPZD1003 and pPZD1004 revealed similar 2.3-kb

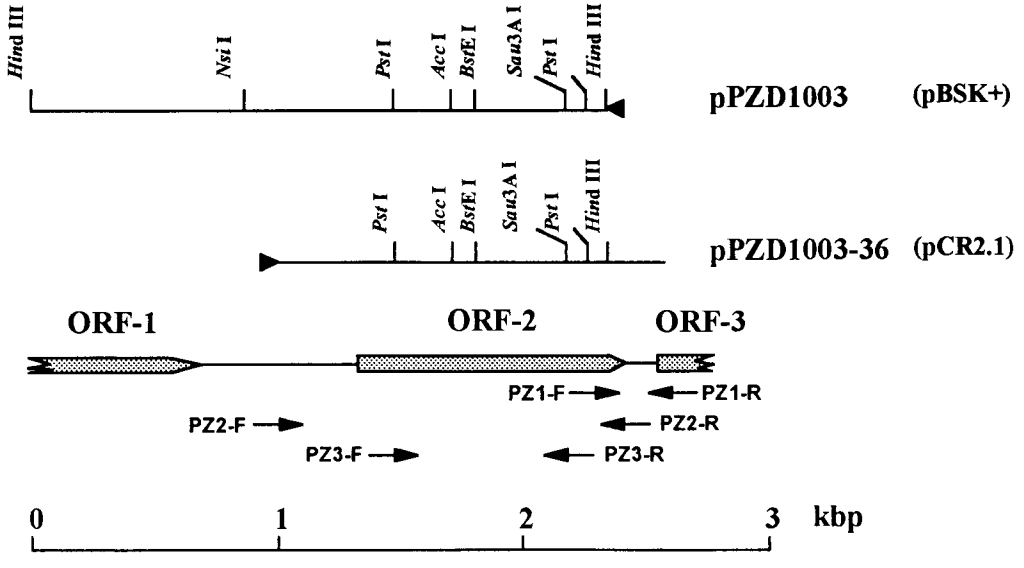

FIG. 1. Restriction maps of plasmids. Small arrows, orientation and location of oligonucleotide primers for PCR amplification and DNA sequencing strategy. Plasmid pPZD1003 contains a 2,322-bp fragment of *B. pilosicoli* strain SP16 chromosomal DNA. Clone pPZD1003-36 contains the full-length ORF-2 of the *B. pilosicoli mglB* gene encoding MglB lipoprotein including a putative promoter region extending 381 bp upstream of the ORF-2 start codon. Large arrows direction of transcription of reading frames. Located 533 bp upstream of ORF-2 is a 5′-truncated ORF-1 encoding the C-terminal region of a polypeptide with 52.4% amino acid sequence similarity with ribosomal large-subunit pseudouridine synthase D encoded by the *B. burgdorferi yfiI* gene. A 3′-truncated ORF-3 encoding a putative *mglA* gene is located 106 bp downstream of ORF-2. Arrowheads, vector promoter.

TABLE 3. Comparison of the predicted amino acid sequence of a polypeptide encoded by ORF-2 of *B. pilosicoli* and recognized by serum IgG antibodies from hyperimmune and convalescent swine with those of MglB lipoproteins of other bacteria

Bacteria	Similarity (%)	Identity (%)	GenBank accession no.
H. influenzae	65.4	56.2	G64096
E. coli	64.6	54.1	M59444
S. enterica serovar Typhimurium	64.0	54.2	S29390
C. freundii	64.3	53.8	X59389
T. pallidum	49.5	39.6	L20301

*Hin*dIII inserts. Therefore only clone pPZD1003 was selected for further sequencing using a double-stranded nested-deletion library strategy.

Sequence analysis. Sequence analysis of clone pPZD1003 revealed a 2,322-bp DNA insert containing two truncated ORFs, designated ORF-1 and ORF-2, separated by 533 bp (Fig. 1). Analysis of the 5′-truncated ORF-1 indicated a nucleotide sequence encoding the C-terminal region of a polypeptide with 52.4% amino acid sequence similarity with ribosomal large-subunit pseudouridine synthase D encoded by the *B. burgdorferi yfiI* gene (16). Analysis of the nucleotide sequence of the 3′-truncated ORF-2 and the predicted amino acid sequence revealed a protein with a high sequence similarity and identity to MglB lipoprotein (Table 3). The missing sequence at the 3′ end of ORF-2 was obtained by sequencing a cloned fragment obtained by PCR amplification of *B. pilosicoli* chromosomal DNA with oligonucleotide primers PZ1-F and PZ1-R (Fig. 1 and Table 2) and cloning into pCR2.1 to create pPZD1003-36 (Fig. 1). Restriction endonuclease analysis of a pPZD1003-36 DNA insert was consistent with the cloned fragment representing the entire ORF-2 sequence (data not shown). ORF-2 consisted of 1,050 bp with an ATG codon at position 441 and a TAA termination codon at position 1,490. The ATG start codon was preceded by two putative promoter sequences (−35 and −10) and a putative ribosomal binding site (Fig. 2). ORF-2 had a G+C content of 34.3% and encoded a putative polypeptide of 349 amino acids with an N-terminal sequence motif corresponding to a leader peptide (the first 21 amino acids) terminated by a signal peptidase II cleavage site suggestive of a lipoprotein (19). The sequence Thr-Val-Ser-Cys in the signal peptide was similar to the Leu-Ala/Ser-Gly/Ala-Cys consensus sequence of a signal peptidase II processing site for lipoprotein with a lipid attachment site located at Cys-21 (Fig. 2). The predicted mature protein was 329 residues in length with a deduced molecular mass of 35.8 kDa. This was consistent with the results of Western blot analysis of pPZD1003-36 reacted with convalescent and hyperimmune swine sera, which indicated a band with an apparent molecular weight of 36,000 (Fig. 3A, lane 2). A band with an apparent molecular weight of approximately 36,000 also was present in a Western blot of *B. pilosicoli* whole-cell lysate reacted with either hyperimmune or convalescent swine sera (Fig. 3A, lane 3). A similar band was not present when these sera were reacted with *E. coli* transformed with pCR2.1 without the DNA insert (Fig. 3A, lane 1). Absorbed sera taken from control swine on day 49 p.i. with sterile medium or from swine prior to challenge or immunization showed no reactivity by Western blot analysis of pPZD1003-36 (data not shown). Further analysis of the cloned PCR products obtained by amplification of *B. pilosicoli* chromosomal DNA with oligonucleotide primers PZ1-F and PZ1-R also revealed a 3′-truncated ORF-3 located 106 bp downstream of ORF-2.

Sequence comparison. The predicted amino acid sequence of the entire polypeptide encoded by ORF-2 had high similarity and identity with MglB of *Haemophilus influenzae*, *E. coli*, *S. enterica* serovar Typhimurium, *Citrobacter freundii*, and *T. pallidum*, as shown by using the BESTFIT alignment program (Table 3). A total of 18 out of 19 amino acids of *E. coli* MglB that interact with galactose (18, 41, 63) were present in the *B. pilosicoli* MglB (Tyr-24, Asp-28, Phe-30, Met-31, Asn-78, Asn-103, Ser-127, His-164, Asp-166, Arg-170, Trp-195, Asn-221, Asn-222, Met-225, Asp-253, Asn-273, Gln-278, and Tyr-313). Pro-124 replaced Ser-112 of *E. coli* MglB (Fig. 4). Analysis of the 260-bp nucleotide sequence comprising the 3′-truncated ORF-3 located 106 bp downstream of ORF-2 revealed 73.8% similarity and 66.3% identity to *mglA* of *E. coli*.

Posttranslational modification of *B. pilosicoli* MglB. Because the deduced amino acid sequence encoded by *B. pilosicoli* ORF-2 indicated a leader peptide terminated by a signal peptidase II cleavage site suggestive of a lipoprotein, incorporation of [^{3}H]palmitate into *E. coli* DH5α transformed with pPZD1003-36 was examined. A strong band with an apparent molecular weight of 36,000 was seen by autoradiography (Fig. 3B, lane 2). A similar band was absent in lysate of *E. coli* transformed with the control plasmid (Fig. 3B, lane 1).

Gene copy number and homologous sequences among intestinal spirochetes. Hybridization of the DIG-labeled recombinant ORF-2 probe (895 bp) carrying more than 90% of the structural gene for the mature lipoprotein with chromosomal DNA from *B. pilosicoli* strain SP16 digested with restriction

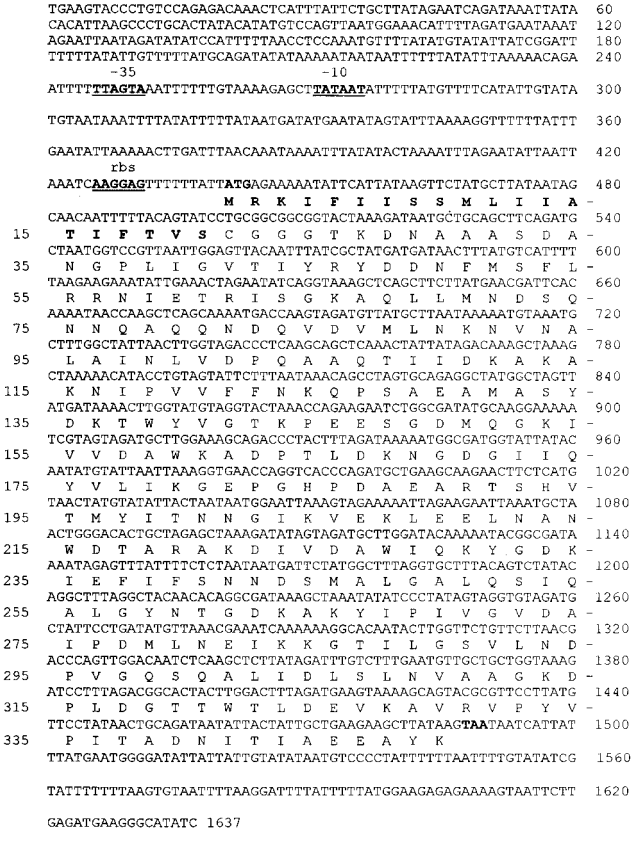

FIG. 2. Sequence of *B. pilosicoli mglB* DNA and corresponding amino acid sequence. Boldface DNA sequences, putative −35 and −10 ribosome-binding sites (rbs), methionine start codon, and termination codon; boldface amino acid sequence, putative leader peptide.

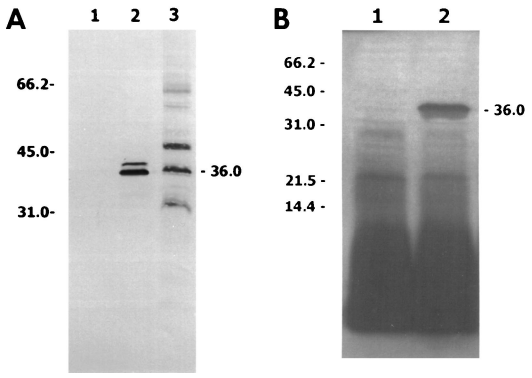

FIG. 3. Western blot (A) and autoradiograph (B) of lipoprotein-labeled recombinant *B. pilosicoli* MglB expressed in *E. coli* DH5α transformed with plasmid pPZD1003-36 or plasmid pCR2.1 as a negative control. (A) *E. coli* DH5α/pPZD1003-36 or *E. coli* DH5α/pCR2.1 control cells grown in LB medium with or without ampicillin (optical density at 600 nm of 0.5), respectively, were separated by SDS–10% PAGE, blotted onto nitrocellulose membranes, and reacted with *B. pilosicoli* hyperimmune swine serum preabsorbed with *E. coli* DH5α, followed by biotin-labeled goat anti-swine IgG, peroxidase-labeled streptavidin, and substrate. Lanes: 1, *E. coli* DH5α/pCR2.1; 2, *E. coli* DH5α/pPZD1003-36; 3, whole-cell lysate of *B. pilosicoli* strain SP16. (B) Same as panel A except that the cells were labeled for 3 h with [^{3}H]palmitate before separation by SDS–12.5% PAGE, drying, and autoradiography. Lanes: 1, *E. coli* DH5α/pCR2.1; 2, *E. coli* DH5α/pPZD1003-36. Molecular weights in thousands are indicated.

enzymes indicated a single copy of the gene. The same probe was hybridized with *Hae*III-digested chromosomal DNA from other intestinal spirochetes (Table 1). Human strain SP16 showed a single band of approximately 12.0 kb, whereas DNA from porcine strains P43/6/78^T and UNL-8 showed bands of approximately 11.0 and 4.2 kb and 10.6 and 1.4 kb, respectively. Southern blot analyses of chromosomal DNA from other intestinal spirochetes with the recombinant ORF-2 probe revealed homologous sequences with different *Hae*III restriction endonuclease digestion patterns in two of three strains of *Serpulina intermedia*, three of four strains of *Brachyspira innocens*, both strains of *Brachyspira murdochii*, and *Brachyspira alvinipulli* but in none of the strains of *B. hyodysenteriae*, *Brachyspira aalborgi*, and *T. succinifaciens* (Table 1). As anticipated, no hybridization signal was present when the probe was reacted with chromosomal DNA from *E. coli* DH5α or DNA from the vector pBSK$^+$.

Amino acid sequence comparison. A comparison of the predicted amino acid sequence of the polypeptide encoded by ORF-2 of *B. pilosicoli* with MglB of *H. influenzae*, *E. coli*, *S. enterica* serovar Typhimurium, *C. freundii*, and *T. pallidum* is presented in Table 3. The nucleotide sequences of *E. coli*, *S. enterica* serovar Typhimurium, and *T. pallidum mglA* are available from the GenBank under accession no. M59444, P23924, and AAC44749, respectively. The accession numbers of the *B. burgdorferi yfiI* gene available from the GenBank are AE001116 and P70870.

DISCUSSION

On the basis of a high amino acid sequence identity with bacterial glucose-galactose transport and chemoreceptor MglB lipoproteins and expression of a recombinant mature lipoprotein with an apparent molecular weight of 36,000, the cloned gene from *B. pilosicoli* was named *mglB* (49). This is consistent with the recent demonstration of a putative *mgl*-like operon in *T. pallidum* (40) and MglB homologues among oral spirochetes *Treponema phagedenis*, *T. denticola*, and *Treponema refringens* (3). A comparison of 19 essential amino acid residues located within the binding site of *E. coli* MglB with the predicted amino acid sequence encoded by ORF-2 revealed 18 identical residues (94.7%), suggesting that the cloned gene from *B. pilosicoli* could encode a glucose-galactose recognition effector of a high-affinity transport system (*mgl* operon). The reason for the higher sequence homology of the *B. pilosicoli mglB* gene with similar genes of *H. influenzae* and *Enterobacteriaceae* than with the corresponding gene of *T. pallidum*, a more phylogenically related spirochete, is unknown (Table 3). Also, the 34.3% G+C content of *mglB* was higher than the 24.6% overall G+C content of *B. pilosicoli* chromosomal DNA (58). Taken together these observations suggest recent acquisition of *mglB* into the genome. Comparing the sequences of the entire *mgl* operons of additional intestinal spirochetes might help clarify this question.

The genetic organization of the *mgl* operons of *E. coli* and *S. enterica* serovar Typhimurium consists of *mglBAC* and *mglBAEC*, respectively (18, 20, and 34). In both bacteria, MglB is a 332-amino-acid protein that functions as the high-affinity (K_m of $\sim 10^{-7}$ M) periplasmic binding protein with a dual function, active import of and chemotaxis towards glucose and galactose (23, 30). Other genes in the operon encode inner membrane proteins MglA, a 506-amino-acid protein with ATPase activity, MglC, a 336-amino-acid protein responsible for the formation of a transmembrane pore, and MglE, a smaller and less-well-characterized protein only present in *S. enterica* serovar Typhimurium (20, 29). Identification of a truncated putative *mglA* gene 106 bp downstream of *mglB* further suggested that *B. pilosicoli* has a gene arrangement within an operon which is similar to those of other bacteria (Fig. 1). Although the dual function of MglB of *E. coli* and *S. enterica* serovar Typhimurium is well established, a similar function in spirochetes remains to be determined.

Susceptibility and resistance to bacterial enteric diseases are multifactorial; however, chemotaxis towards mucin appears to play a key role in the pathogenesis of bacterially induced enteric infections (1, 21, 24, 26, 31, 33). Mucin is the principal

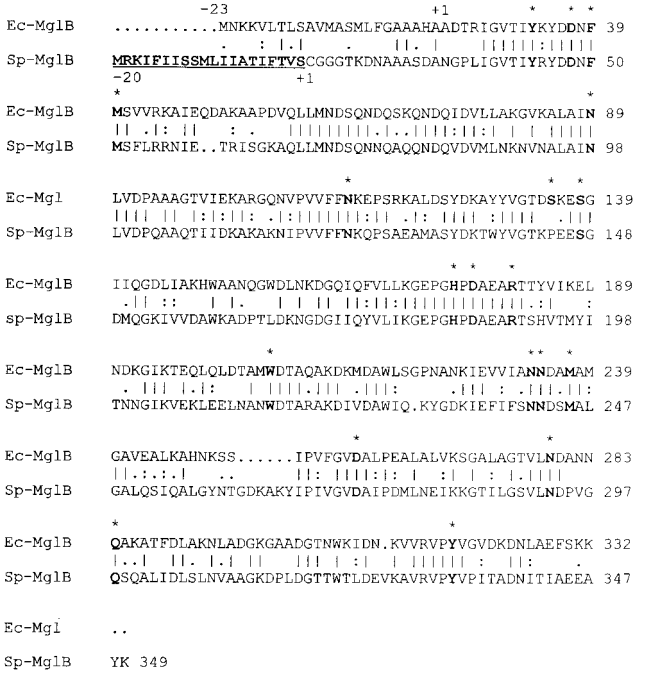

FIG. 4. BESTFIT alignment of *B. pilosicoli* (Sp) and *E. coli* (Ec) MglB. Vertical dash, identity between sequences; two dots, conservative substitution; one dot, acceptable substitution; asterisks, 19 amino acids which form the carbohydrate-binding site of MglB.

constituent of the colonic mucus gel and consists of complex glycoproteins synthesized and secreted by goblet cells (50). The primary function of intestinal mucin is to provide a selective diffusion barrier against penetration of the mucosa by bacteria, toxins, and dietary components, but mucin degradation and utilization by the intestinal bacterial flora are well established (5, 21, 26, 31). In addition to the inherent enhanced motility of spirochetes in viscous materials, chemotaxis towards mucin appears to be important in the mucosal localization of *B. hyodysenteriae* (24, 33) and *B. pilosicoli* (64). Because galactose, galactosamine, and glucosamine together make up half of the pig colonic mucin glycoproteins (32) and because D-glucose, D-galactose, *N*-acetyl-D-glucosamine, and D-glucosamine are utilized by *B. pilosicoli* as substrates for growth in vitro (58, 60), a role for MglB in mucosal localization of *B. pilosicoli* would be consistent with the biology of this spirochete. This is also consistent with specific uptake of radiolabeled glucosamine, but not sulfate and fucose, by spirochetes attached along the rectal mucosal epithelia of patients with CS (37). Therefore, the ability of *B. pilosicoli* to penetrate the highly viscous colonic mucin together with chemotaxis towards mucin, perhaps through MglB-mediated sensory transduction mechanisms, may facilitate translocation from the lumen to the epithelial surface for establishment of intimate attachment (35, 36, 48, 54, 56, 57). However, the presence of homologous sequences in closely related but nonpathogenic commensals *B. innocens* and *B. murdochii* suggests that other virulence determinants also may be required for pathogenesis.

On the basis of the present observations, swine that recovered from CS developed serum IgG antibodies specific for a putative MglB lipoprotein. This is consistent with *T. pallidum* infection in human beings, in which a serum antibody response to a putative MglB lipoprotein antigen is seen (42, 47, 48). However, this is not surprising considering that spirochetal lipoproteins are highly immunogenic antigens and elicit a strong antibody response (4, 43).

Colonic spirochetosis is important to the swine industry because of the economic impact of the disease on pig production. Conversely, infection of humans with *B. pilosicoli* has been found in the developing world and among immunocompromised individuals in developed countries. Although it remains unclear whether CS is a zoonotic disease, the structural, biochemical, genotypic, and pathogenetic characteristics of *B. pilosicoli* isolated from human beings are similar to those of *B. pilosicoli* isolated from animals, and the disease of swine can be used as a model to study the pathogenesis of human CS. Although most of the genes and proteins involved in motility and chemotaxis, including the *mgl* operons of other enteric and food-borne pathogens of humans, have been characterized, nothing is known about their role in colonization of the gut and pathogenesis of disease. While the *mgl* operon may facilitate motility and/or colonization of the colonic mucosa by *B. pilosicoli*, it is either distantly related or not required for colonization and disease caused by *B. hyodysenteriae* and *B. aalborgi*. There is a fundamental need to understand the basic mechanism(s) involved in intestinal bacterial colonization and its relationship to the induction of disease. A better understanding of the role of sensory transduction events associated with colonization of the colonic mucosa by *B. pilosicoli* could provide a molecular basis for the development of more-effective strategies for prevention of CS.

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