JOURNAL OF BACTERIOLOGY, Dec. 1992, p. 7729–7742
0021-9193/92/237729-14$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 174, No. 23

Cloning and Sequencing of a Gene Encoding a 21-Kilodalton Outer Membrane Protein from *Bordetella avium* and Expression of the Gene in *Salmonella typhimurium*

CLAUDIA R. GENTRY-WEEKS,[1,2]* ANNE-LISE HULTSCH,[2] SANDRA M. KELLY,[2] JERRY M. KEITH,[1] AND ROY CURTISS III[2]

Laboratory of Microbial Ecology, National Institute of Dental Research, Bethesda, Maryland 20892,[1] and Department of Biology, Washington University, St. Louis, Missouri 63130[2]*

Received 27 May 1992/Accepted 4 October 1992

Three gene libraries of *Bordetella avium* 197 DNA were prepared in *Escherichia coli* LE392 by using the cosmid vectors pCP13 and pYA2329, a derivative of pCP13 specifying spectinomycin resistance. The cosmid libraries were screened with convalescent-phase anti-*B. avium* turkey sera and polyclonal rabbit antisera against *B. avium* 197 outer membrane proteins. One *E. coli* recombinant clone produced a 56-kDa protein which reacted with convalescent-phase serum from a turkey infected with *B. avium* 197. In addition, five *E. coli* recombinant clones were identified which produced *B. avium* outer membrane proteins with molecular masses of 21, 38, 40, 43, and 48 kDa. At least one of these *E. coli* clones, which encoded the 21-kDa protein, reacted with both convalescent-phase turkey sera and antibody against *B. avium* 197 outer membrane proteins. The gene for the 21-kDa outer membrane protein was localized by Tn*5*seq1 mutagenesis, and the nucleotide sequence was determined by dideoxy sequencing. DNA sequence analysis of the 21-kDa protein revealed an open reading frame of 582 bases that resulted in a predicted protein of 194 amino acids. Comparison of the predicted amino acid sequence of the gene encoding the 21-kDa outer membrane protein with protein sequences in the National Biomedical Research Foundation protein sequence data base indicated significant homology to the OmpA proteins of *Shigella dysenteriae*, *Enterobacter aerogenes*, *E. coli*, and *Salmonella typhimurium* and to *Neisseria gonorrhoeae* outer membrane protein III, *Haemophilus influenzae* protein P6, and *Pseudomonas aeruginosa* porin protein F. The gene (*ompA*) encoding the *B. avium* 21-kDa protein hybridized with 4.1-kb DNA fragments from *Eco*RI-digested, chromosomal DNA of *Bordetella pertussis* and *Bordetella bronchiseptica* and with 6.0- and 3.2-kb DNA fragments from *Eco*RI-digested, chromosomal DNA of *B. avium* and *B. avium*-like DNA, respectively. A 6.75-kb DNA fragment encoding the *B. avium* 21-kDa protein was subcloned into the Asd$^+$ vector pYA292, and the construct was introduced into the avirulent Δ*cya* Δ*crp* Δ*asd S. typhimurium* χ3987 for oral immunization of birds. The gene encoding the 21-kDa protein was expressed equivalently in *B. avium* 197, Δ*asd E. coli* χ6097, and *S. typhimurium* χ3987 and was localized primarily in the cytoplasmic membrane and outer membrane. In preliminary studies on oral inoculation of turkey poults with *S. typhimurium* χ3987 expressing the gene encoding the *B. avium* 21-kDa protein, it was determined that a single dose of the recombinant *Salmonella* vaccine failed to elicit serum antibodies against the 21-kDa protein and challenge with wild-type *B. avium* 197 resulted in colonization of the trachea and thymus with *B. avium* 197.

Bordetella avium is the etiological agent of bordetellosis of turkeys and chickens (7, 40, 44, 65, 66). Bordetellosis in birds is a localized infection of the upper respiratory tract (7, 40, 61, 66) which closely resembles whooping cough in humans (46). *B. avium* initiates infection in birds by attaching to the ciliated epithelial cells which extend from the nasal cavity to the primary bronchi of the lung (3, 4, 33, 48, 72). Recently, it has been determined that in addition to the upper respiratory tract, *B. avium* colonizes the thymus of infected birds (17).

Although the roles of flagella and fimbriae in bordetellosis of birds have been thoroughly investigated (1, 23, 43), the outer membrane proteins of *B. avium*, including those responsible for the structural integrity of the cell and adherence to the ciliated respiratory epithelium, have not been fully characterized. Previous investigators compared the adherence of *B. avium* with that of *B. avium*-like bacteria and Tn*5*-generated *B. avium* mutants in birds in order to identify potential adhesins (5). The results from these studies suggested that a guinea pig hemagglutinin and additional protein(s) play a role in adherence of *B. avium* to the trachea. However, these experiments did not definitively identify the outer membrane protein(s) responsible for prolonged colonization of younger birds, since the in vivo adherence assays were performed with 22-day-old turkeys and spanned a limited period of 1 h.

Comparison of the outer membrane protein profiles of *B. avium*-like bacteria and *B. avium* 75-ura (a Tn*5*-generated *B. avium* auxotroph with reduced hemagglutinating activity) with that of wild-type *B. avium* indicated that a number of their outer membrane proteins differed in molecular mass but failed to identify a specific protein which correlated with adherence (39). Sera and tracheal washings from *B. avium*-infected turkeys contained antibody against outer membrane proteins with molecular masses of 100, 97, 55, 36, 31, 21, 18, and 14 kDa (38). Antibody against the 21-kDa outer membrane protein was elicited at a higher level than antibodies against other *B. avium* outer membrane proteins over a 3-week convalescent-phase period, suggesting that this protein could serve as a protective antigen.

The role of several environmentally regulated and nonreg-

* Corresponding author.

ulated outer membrane proteins in the pathogenicity and structural integrity of *B. avium* is unknown. In this study, we cloned five outer membrane protein genes of *B. avium* 197 in an attempt to begin to determine the role of individual outer membrane proteins in bordetellosis of birds. Our interests focused primarily on the gene encoding the 21-kDa protein, since studies in our laboratory and by others (38) indicated that this protein was immunogenic and might be a vaccine candidate. The gene encoding the *B. avium* 21-kDa protein was cloned, sequenced, and introduced and expressed in an avirulent *Salmonella typhimurium* strain in order to construct a bivalent, oral vaccine for immunization of turkeys. The results of preliminary experiments using the *S. typhimurium* vaccine construct for induction of an immune response against the 21-kDa *B. avium* protein are reported.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and media. The bacterial strains, cosmid and plasmid vectors, and recombinant clones constructed in this laboratory are listed in Table 1. Cosmid pYA2329 was constructed by ligating the 1.8-kb *Sac*II-*Kpn*I-digested, T4 DNA polymerase-treated DNA fragment conferring spectinomycin resistance from pUCD2 (15) to *Sal*I-digested, Klenow fragment-treated pCP13 (20).

All *Escherichia coli* strains, except χ6097, were grown in Luria-Bertani (LB) medium (47) supplemented with (per milliliter) either 25, 50, or 150 μg of tetracycline, spectinomycin, or ampicillin, respectively, for plasmid maintenance. *E. coli* χ6097 and *S. typhimurium* χ3730 and χ3987 were grown in LB medium containing 50 μg of diaminopimelic acid per ml. For production of bacteriophage P22HT *int* lysates, *S. typhimurium* χ3730(pYA2336) was grown in LB broth supplemented with 0.05% galactose prior to bacteriophage infection.

B. avium and *B. avium*-like strains were grown in modified Stainer-Scholte medium supplemented with 0.2% 2-ketoglutarate, 0.2% pyruvate, pantothenate (10 μg/ml), L-phenylalanine (20 μg/ml), and nicotinamide (500 μg/ml); *Bordetella bronchiseptica* and *Bordetella pertussis* strains were grown in modified Stainer-Scholte medium as described previously (28).

Reagents. Restriction enzymes used throughout this study were obtained from Bethesda Research Laboratories, Gaithersburg, Md. All chemicals were obtained from Sigma Chemical Company, St. Louis, Mo. Polyclonal rabbit serum against *B. avium* 197 outer membrane proteins was produced as described previously (29). Convalescent-phase turkey serum was obtained by infection of turkey poults with *B. avium* 197. Four-day-old Nicholas turkey poults were contact-infected by exposure to a 30-day-old turkey that had been infected at 2 days of age with *B. avium* 197. Forty days postexposure, the turkey poults were anesthetized by inhalation of methoxyflurane (Metafane; Pitman-Moore, Mundelein, Ill.) and exsanguinated to obtain convalescent-phase sera.

Construction of *B. avium* 197 gene libraries in *E. coli* LE392. Chromosomal DNA was isolated from *B. avium* 197 by the method of Hull and coworkers (42). *B. avium* 197 DNA was partially digested with either *Sau*3a, *Hin*dIII, or *Xho*I and size-fractionated by sucrose gradient centrifugation, and 25- to 27-kb *Sau*3a-*Hin*dIII-, or *Xho*I-generated DNA fragments were selected for ligation. The size-fractionated, *Sau*3a-digested *B. avium* 197 DNA was ligated to *Bam*HI-digested cosmid pYA2329 DNA, while the size-fractionated, *Hin*dIII- and *Xho*I-digested *B. avium* 197 DNA was ligated to *Hin*dIII- and *Xho*I-digested pCP13 DNA, respectively. The ligated DNA was in vitro packaged into lambda phage head and tail proteins supplied by the Packagene kit (Promega Biotec, Madison, Wis.) and transfected into *E. coli* LE392 (69) by using the manufacturer's instructions. Recombinant *E. coli* clones were plated onto LB agar containing 50 μg of either tetracycline or spectinomycin per ml.

Subcloning of genes encoded by recombinant cosmids and restriction enzyme mapping were performed according to established procedures (47).

Immunological screening of *B. avium* 197 gene libraries. Recombinant *E. coli* clones were tested for reactivity with antibody against *B. avium* 197 outer membrane proteins and convalescent-phase sera from *B. avium* 197-infected turkeys by the colony immunoblot assay as described previously (28).

An identical protocol was used to identify *E. coli* recombinant clones which produce *B. avium* 197 proteins which react with convalescent-phase turkey serum, except that convalescent-phase turkey serum was used as the primary antibody and horseradish peroxidase-labeled goat anti-turkey immunoglobulin G antibody (Organon Teknika-Cappel, West Chester, Pa.) was used as the secondary antibody.

Western immunoblot of *B. avium* 197 proteins produced by *E. coli* recombinant clones. Proteins from *E. coli* recombinant clones were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of boiled whole-cell proteins, electrotransfer of proteins to nitrocellulose filters, and Western immunoblot analysis as described previously (28, 71). The percentage of SDS-polyacrylamide gel varied from 10 to 12.5% throughout this study to allow optimum resolution of the protein of interest.

Transposon mutagenesis of the gene encoding the *B. avium* 21-kDa outer membrane protein. A 6.75-kb *Bam*HI-*Pst*I DNA fragment from pYA2320 was subcloned into *Bam*HI-*Pst*I-digested pUC8-1 (35), generating pWDC371. The construct pWDC371 was transformed by standard methods (47) into *E. coli* CC118::Tn*5*seq1 for transposon mutagenesis (41, 54). Briefly, *E. coli* CC118::Tn*5*seq1 containing pWDC371 was inoculated into LB broth containing 250 μg of neomycin per ml and incubated for 3 days at 37°C. Plasmid DNA was isolated from the culture and transformed into *E. coli* HB101 (8), and *E. coli* cells containing plasmids with transposon insertions were selected by plating on LB agar containing 50 μg of kanamycin per ml. *E. coli* transformants containing Tn*5*seq1-mutated pWDC371 were tested for loss of reactivity with antibody against *B. avium* 197 outer membrane proteins by the colony immunoblot assay described above. Plasmid DNA was purified from *E. coli* transformants which lacked reactivity with the antibody by using reagents from the Qiagen kit (Qiagen, Inc., Studio City, Calif.) or by alkaline lysis purification and cesium chloride gradient ultracentrifugation (47). Restriction enzyme analysis of the plasmid DNA was performed according to established procedures (47).

DNA sequence and analysis. Plasmid pWDC371 DNA containing Tn*5*seq1 insertions in the *ompA* gene were subjected to DNA sequence analysis. Sequencing reactions were performed by the Sanger dideoxy chain termination method (62) by using the reagents of the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). The T7 and SP6 sequencing primers were obtained from Pharmacia LKB Biotechnology, Piscataway, N.J., and New England Biolabs, Inc., Beverly, Mass., respectively. Polyacrylamide sequencing gel solution (Gel-

TABLE 1. Bacterial strains, vectors, and recombinant clones

Designation (enzyme/vector or DNA fragment used for construction; protein encoded)	Relevant genotype or phenotype	Origin or reference
B. avium		
197	Wild type; virulent for turkeys	Y. M. Saif, Ohio
002 isolate B	Virulent for turkeys	Y. M. Saif, Ohio
27/83-T1	Virulent for turkeys	H. A. Berkhoff, N.C.
838	Virulent for turkeys	M. S. Hofstad, Iowa
4671	Virulent for turkeys	D. G. Simmons, N.C.
105	Virulent for turkeys	J. K. Skeeles, Ark.
B. avium-like 031	Avirulent for turkeys	Y. M. Saif, Ohio
B. bronchiseptica 469	Virulent for dogs and swine	E. Tuomanen, N.Y.
B. pertussis		
Tohama I	Virulent for humans	W. E. Goldman, Mo.
BB114	Virulent for humans	W. E. Goldman, Mo.
E. coli K-12		
LE392	F^- *hsdR514* (r_K^- m_K^+) *supE44 supF58 lacY1* or Δ(*lacIZY*)6 *galK2 galT22 metB1 trpR55* λ^-	69
χ6097	F^- *ara* Δ(*pro-lac*) *rpsL* Δ*asd-4* Δ[*zhf-2*::Tn*10*] *thi* ϕ80d*lacZ*ΔM15	55
CC118::Tn*5*seq1	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE*(Am) *recA1* Tn*5*seq1	41, 54
HB101	F^- *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* λ^-	8
S. typhimurium		
χ3730	*leu hsdLT galE trpD2 rpsL20* Δ*asdA1* Δ[*zhf-4*::Tn*10*] *metE551 metA22 hsdSA hsdSB ilv*	This work
χ3761	Chicken-passaged strain 30875 (contains 91-kb pStV1 plasmid)	70
χ3985	$pStV1^+$ Δ*cya-12* Δ*crp-11* derivative of χ3761	19
χ3987	$pStV1^+$ Δ*cya-12* Δ*crp-11* Δ*asdA1*	This work
Bacteriophage P22HT*int*	Increased frequency of generalized transduction	64
Cosmid		
pCP13	Mob^+ Tra^- Tc^r Km^r, low-copy-number, broad-host-range vector	20
pYA2329	Derivative of pCP13 Mob^+ Tra^- Sp^r Km^r	This work
Plasmid		
pYA292	*S. typhimurium asd* selection marker, P*trc* promoter, multiple cloning site in *lacZ*(α), transcription terminators.	27
pUC8-1	Ap^r, high-copy-number, P*lac* promoter, multiple cloning site in *lacZ*(α).	35
Recombinant cosmids		
pYA2320 (*Xho*I/pCP13; 21kDa)		This work
pYA2326 (*Hin*dIII/pCP13; 40 kDa)		This work
pYA2333 (*Sau*3a/pYA2329; 56 kDa)		This work
pYA2337 (*Sau*3a/pYA2329; 38 kDa)		This work
pYA2338 (*Sau*3a/pYA2329; 43 kDa)		This work
pYA2339 (*Sau*3a/pYA2329; 48 kDa)		This work
pYA2336 (*Bam*HI-*Pst*I/pYA292; 21kDa)		This work
Recombinant plasmid pWDC371 (6.75-kb *Bam*HI-*Pst*I DNA fragment from pYA2320 subcloned into pUC8-1; 21 kDa)		This work

Mix 8) was obtained from Bethesda Research Laboratories, and ^{35}S-deoxyadenosine 5'-[α thio]triphosphate (^{35}S-dATP; 1,000 to 1,500 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

DNA sequence analysis was performed with sequence analysis software programs from the Genetics Computer Group, Inc., Madison, Wis., and the MacVector sequence analysis software (International Biotechnologies, Inc., New Haven, Conn.).

Southern hybridization of *Bordetella* chromosomal DNA with the gene encoding the 21-kDa outer membrane protein. Chromosomal DNA was isolated from *B. avium* 002 isolates B, 27/83-T1, 838, 4671, and 105, *B. avium*-like 031, *B. pertussis* Tohama I and BB114, and *B. bronchiseptica* 469 by using the method of Hull and coworkers (42).

The DNA probe used for hybridization was a polymerase chain reaction (PCR)-generated internal fragment of the gene encoding the 21-kDa outer membrane protein which spanned nucleotides 223 to 710 (see Fig. 4) and consisted of 487 bp. The primers used for PCR synthesis of the probe were 5'-AACAAACCCTCCAAATTCGCTCTGGCGCTTGCCTTCGC-3' and 5'-TTGCCTTCCGTGTAGCTGCAGTTCGGATCGATGCCCTTGC-3'. The 487-bp DNA fragment was synthesized with the DNA Thermal Cycler machine (Perkin-Elmer Cetus, Norwalk, Conn.) by using reagents and instructions in the Gene Amp DNA Amplification Reagent kit (Perkin-Elmer Cetus). Formamide (Fluka Chemie AG, Buchs, Switzerland) was included at a final concentration of 10% in the PCR to increase specificity (63). Following PCR synthesis, the 487-bp DNA fragment was purified by agarose gel electrophoresis and subsequent elution by using reagents from the GENECLEAN kit (BIO 101, La Jolla, Calif.). The 487-bp DNA fragment was radiolabelled by Lofstrand Laboratories Ltd., Gaithersburg, Md., by incorporation of ^{32}P-dATP by the random primer labeling reaction.

Chromosomal DNA from the *Bordetella* strains listed above was digested with *Eco*RI, electrophoresed through a 0.6% agarose gel, and transferred to GeneScreen Plus hybridization membrane (New England Nuclear Corp.) according to the manufacturer's instructions. The membrane was prehybridized for 1 h at 55°C in 1% SDS–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–300-μg/ml denatured salmon sperm DNA (Sigma Chemical Co.). The ^{32}P-labeled, DNA probe was added directly to the prehybridization solution at a concentration of 2.86×10^5 cpm/ml and incubated overnight at 55°C. The membrane was washed according to the manufacturer's instructions at 65°C. The membranes were exposed to X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.) with two intensifying screens for 24 h at −70°C and developed with a Kodak M35A X-Omat processor.

Construction of an *S. typhimurium* bivalent vaccine strain expressing the gene encoding the 21-kDa *B. avium* outer membrane protein. *S. typhimurium* χ3987 was the bacterial host used to construct a bivalent vaccine for oral immunization of turkeys with the 21-kDa outer membrane protein of *B. avium*. The *S. typhimurium* χ3987 parent strain, χ3761, was an equine isolate which was passaged through chickens and reisolated from the spleens of infected birds (Table 1). Deletion mutations in the *cya* and *crp* genes were introduced via classical genetic methods (18) into *S. typhimurium* χ3761 to yield χ3985. *S. typhimurium* χ3985 is avirulent in day-old chicks and has been shown to protect immunized chickens from colonization with the wild-type χ3761 (19, 36). In addition, a deletion was introduced into the *asd* gene of the avirulent Δ*cya* Δ*crp* *S. typhimurium* χ3985 (55) (generating strain χ3987) to allow stable maintenance of the Asd$^+$ plasmid pYA292. The *B. avium* gene for the 21-kDa protein was introduced into *S. typhimurium* χ3987 via a restriction-deficient, modification-proficient strain, *S. typhimurium* χ3730. A 6.75-kb *Bam*HI-*Pst*I *B. avium* DNA fragment from the recombinant cosmid pYA2320 was ligated to *Bam*HI-*Pst*I-digested pYA292 (27) and transformed into Δ*asd E. coli* χ6097 (55). The resulting plasmid, designated pYA2336, encoded the gene encoding the 21-kDa protein and was transformed into *S. typhimurium* χ3730 by standard methods (47). Bacteriophage P22HT*int* (64) was propagated on log-phase *S. typhimurium* χ3730(pYA2336) as previously described (21), and the bacteriophage lysate was used to transduce pYA2336 into *S. typhimurium* χ3987. Transductants were selected on LB agar, and the presence of plasmid pYA2336 DNA was verified by DNA isolation and restriction enzyme analysis. Expression of the *B. avium* 21-kDa protein gene in *E. coli* χ6097 and *S. typhimurium* χ3987 was confirmed by Western blot analysis using antibodies against *B. avium* outer membrane proteins as described above.

Localization of the *B. avium* 21-kDa outer membrane protein in *S. typhimurium* χ3987(pYA2336). *S. typhimurium* χ3987(pYA292) and *S. typhimurium* χ3987(pYA2336) cells were lysed and fractionated into inner membrane, outer membrane, and soluble fractions according to the method of Tanji and coworkers (68).

Crude cell lysates and inner membrane, outer membrane, and soluble fractions were mixed with an equal volume of sample buffer, boiled for 5 min, and subjected to SDS-PAGE through 4 to 20% polyacrylamide gradient gels (Schleicher & Schuell, Inc., Keene, N.H.). Proteins were transferred to nitrocellulose filters (Schleicher & Schuell, Inc.) and reacted with antibody against *B. avium* 197 outer membrane proteins as described above.

Immunization of turkeys with *S. typhimurium* χ3987 (pYA2336) and challenge with *B. avium* 197. Bacterial cultures for oral inoculation of turkeys were prepared as described previously (18). Three groups of 15 1-day-old Nicholas turkey poults (Western Turkey Eggs, Temple, Tex.) were orally inoculated with a 100-μl inoculum of either 1.5×10^9 CFU of *S. typhimurium* χ3987(pYA2336) or 2.9×10^9 CFU of *S. typhimurium* χ3987(pYA292) or buffered saline gelatin (18). Nine days later, all three groups of birds were challenged intranasally and ocularly with 1.2×10^8 and 6×10^7 CFU of *B. avium* 197, respectively. At 4, 7, 10, and 18 days postchallenge, three birds from each group were necropsied. Blood was collected from all birds at the time of necropsy, and serum was collected and stored at −70°C. Birds were euthanized by CO_2 asphyxiation or by cardiac embolism. At the initiation of the bird immunization experiments, several birds in each group died as the result of a nonrelated, osteomyelitis-like illness. Since this reduced the number of birds per group, three birds were necropsied at each time point until no birds remained.

For assay of the humoral immune response of immunized birds, either whole *B. avium* 197 or *S. typhimurium* χ3987(pYA2336) cells were boiled for 10 min in sample buffer, applied to an SDS–12.5% polyacrylamide gel with a continuous well, and electrophoresed to attain separation of the proteins. The separated proteins were transferred to nitrocellulose filters as described above. The filters were assembled into a Mini-PROTEAN II Multiscreen apparatus (Bio-Rad Laboratories, Richmond, Calif.) for simultaneous screening of the individual serum samples obtained from the birds. The serum samples from birds immunized with either *S. typhimurium* χ3987(pYA292) or *S. typhimurium* χ3987

(pYA2336) and challenged with *B. avium* 197 were diluted 1:100 in NET buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 5 mM EDTA, 0.25% gelatin, 0.05% Triton X-100) and applied to the individual channels of the Multiscreen apparatus. Serum samples from nonimmunized, nonchallenged birds and sera from nonimmunized, *B. avium* 197-challenged birds were diluted 1:100 in NET buffer and served as negative control sera. Rabbit antibody against *B. avium* 197 outer membrane proteins (described above) served as a positive control and was diluted 1:1,000 in NET buffer and applied to a channel of the apparatus. The primary antibody was incubated with the filters for 24 h at room temperature. After the channels were washed with NET buffer, horseradish peroxidase-labeled goat anti-turkey immunoglobulin G (diluted 1:500 in NET buffer) (Organon Teknika-Cappel) and horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G (diluted 1:800 in NET buffer) (ICN Biomedicals) were added to the channels exposed to turkey primary antibody and rabbit primary antibody, respectively. Following incubation, the nitrocellulose filters were washed with 10 mM Tris, pH 7.5, and developed with 4-chloro-1-naphthol.

Colonization and persistence of Δ*cya* Δ*crp* Δ*asd S. typhimurium* with either pYA292 or pYA2336 were determined at 13, 16, 19, and 27 days after oral *S. typhimurium* inoculation by enumerating the bacteria in representative samples of the spleen, bursa, thymus, and contents and wall of the small intestine. A sample of spleen weighing less than 0.5 g was removed from each bird and placed in a preweighed polypropylene tube (17 by 100 mm). The exact weight of the tissue sample was determined, 9 volumes of chilled buffered saline gelatin was added, and the sample was placed on ice. Representative samples of the bursa of Fabricius, small intestine wall, and small intestine contents were taken and treated as described above to enumerate CFU per gram of sample.

Tracheal titers of *B. avium* 197 were obtained by removing a 3-cm section of trachea from each turkey, placing the section in a preweighed polypropylene tube, determining the weight of the tissue, adding 9 volumes of chilled buffered saline gelatin, and placing the samples on ice. Recovery of *B. avium* 197 from the thymus was determined by removing a sample of less than 0.5 g and treating the tissue as described above. All tissue samples were homogenized with a Brinkman (Westbury, N.Y.) homogenizer as described previously (28) and placed on ice. The homogenizer probe was decontaminated between samples by running the probe for 30 s in a 0.25% Amphyl (National Laboratories, Montvale, N.J.) solution and then for 30 s in 70% ethanol and then rinsing in deionized water. Undiluted samples (100 μl) and appropriate dilutions of the tracheal and thymus homogenates were plated on Penassay agar containing 12.5 μg of tetracycline per ml, while undiluted samples (100 μl) and appropriate dilutions of the spleen and bursa and the small intestine and its contents were plated on MacConkey agar supplemented with maltose (final concentration, 1.0%) and 50 μg of diaminopimelic acid per ml.

Nucleotide sequence accession number. The GenBank accession number for the gene encoding the 21-kDa outer membrane protein is M96550.

RESULTS

Recombinant *E. coli* clones expressing genes encoding *B. avium* 197 outer membrane proteins. Three recombinant libraries were constructed in *E. coli* LE392 with *B. avium* 197 chromosomal DNA which was partially digested with either *Sau*3a, *Hin*dIII, or *Xho*I. Five recombinant *E. coli* clones which expressed genes encoding *B. avium* 197 outer membrane protein were identified by reactivity with antibody against *B. avium* 197 outer membrane proteins (Fig. 1). One *E. coli* clone isolated from the *Xho*I-generated cosmid library produced a 21-kDa protein, and the recombinant cosmid was designated pYA2320. *E. coli* LE392(pYA2326) was isolated from the *Hin*dIII-generated cosmid library and specified a 40-kDa protein. Three recombinant clones, containing cosmids pYA2337, pYA2338, and pYA2339, were isolated from the *Sau*3a-generated cosmid library and encoded the genes for 38-, 43-, and 48-kDa proteins, respectively. *E. coli* LE392(pYA2320) reacted with both antibody against *B. avium* 197 outer membrane proteins and turkey convalescent-phase sera (Fig. 2).

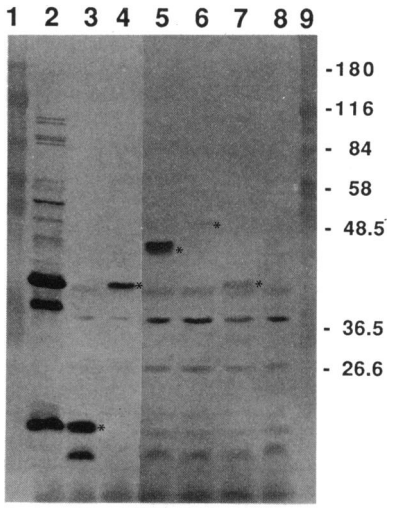

FIG. 1. Western blot of proteins produced by *E. coli* recombinant clones expressing genes encoding *B. avium* outer membrane proteins. Proteins from whole cells were separated by SDS-PAGE (10% polyacrylamide), transferred to a nitrocellulose filter, and reacted with antibody against *B. avium* 197 outer membrane proteins. Lanes: 1 and 9, molecular weight standards; 2, *B. avium* 197 whole cells; 3, *E. coli* LE392(pYA2320); 4, *E. coli* LE392(pYA2337); 5, *E. coli* LE392(pYA2338); 6, *E. coli* LE392(pYA2339); 7, *E. coli* LE392(pYA2326); 8, *E. coli* LE392(pYA2329). Asterisks indicate relevant protein bands.

One *E. coli* recombinant clone from the *Sau*3a-generated library was identified by reactivity with convalescent-phase turkey sera. This recombinant *E. coli* produced a 56-kDa protein and contained the recombinant cosmid pYA2333 (data not shown).

Localization and DNA sequence analysis of the gene encoding the 21-kDa outer membrane protein. A series of Tn*5*seq1 insertions which inactivated the gene encoding the 21-kDa outer membrane protein were obtained by transposon mutagenesis of pWDC371. Restriction enzyme analysis of Tn*5*seq1-mutagenized pWDC371 localized the gene encoding the 21-kDa protein within a 2.49-kb *Bam*HI-*Nru*I DNA fragment. The DNA sequence of the *B. avium* 21-kDa outer membrane protein gene was determined by bidirectional sequencing with T7 and SP6 primers complementary to the ends of Tn*5*seq1. A partial restriction map and the sequencing strategy are shown in Fig. 3. Translation of the DNA sequence between nucleotides 220 and 804 resulted in an open reading frame for a protein of 194 amino acids with a

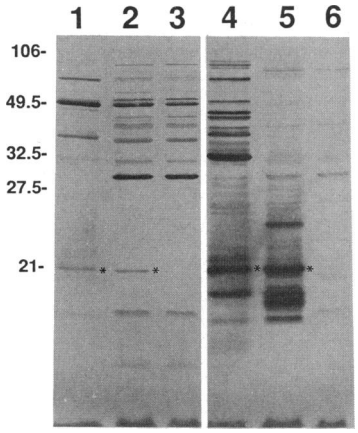

FIG. 2. Reactivity of the *E. coli* clone expressing the gene encoding the *B. avium* 21-kDa protein with both turkey convalescent-phase sera and rabbit antibody against *B. avium* 197 outer membrane proteins in the Western blot assay. Lanes: 1, *B. avium* 197 whole cells; 2, *E. coli* LE392(pYA2320); 3, *E. coli* LE392 (pYA2329); 4, *B. avium* 197 whole cells; 5, *E. coli* LE392(pYA2320); 6, *E. coli* LE392(pYA2329). Proteins were electrophoresed through an SDS–10% polyacrylamide gel prior to transfer to nitrocellulose filters. Lanes 1 to 3 were reacted with turkey convalescent-phase sera, while lanes 4 to 6 were reacted with rabbit antibody against *B. avium* 197 outer membrane proteins. Asterisks designate the 21-kDa protein.

deduced molecular weight of 21,113 and a calculated pI of 9.5 (Fig. 4). The 21-kDa protein contains a signal peptide with probable cleavage sites after amino acid 22 or 24, which follow the "(−3, −1) rule" for signal peptidase cleavage (73). Processing of the protein at either site would result in a mature protein with a molecular weight of either 18,966 or 18,808. The putative signal peptide of the 21-kDa protein is composed of a region of uncharged amino acids followed by a core of hydrophobic residues with alternating neutral residues (Fig. 4) and corresponds to signal peptides of exported bacterial proteins (57).

A putative promoter region spans nucleotides 117 to 122 (TTGAAC) and 139 to 144 (TATACT) and closely conforms to the consensus sequences of promoters recognized by *E. coli* sigma 32 RNA polymerases (Fig. 4) (60). There are 16

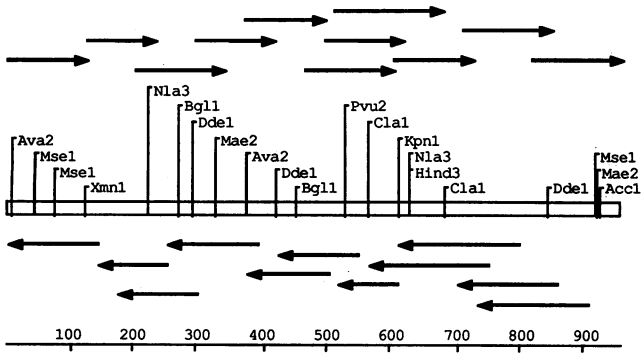

FIG. 3. Restriction map and strategy for sequencing the gene encoding the *B. avium* 21-kDa protein. Arrows indicate the positions of the Tn*5*seq1 insertions used for sequencing and the areas of the gene which were sequenced. The units of the ruler below the restriction map indicate base pairs of DNA.

bases between the −35 and −10 regions of the putative promoter, and such spacing is optimal for expression of the gene in *E. coli* (60). A putative Shine-Dalgarno sequence (GAGG) is located 6 bases upstream of the ATG translation initiation codon. The Shine-Dalgarno sequence and the location upstream from the ATG codon agree with the preferred spacing and nucleotide sequence of ribosomal binding sites in *E. coli* genes (31). A stem-loop structure which consists of a 13-base stem separated by a 3-base loop is located 22 bases downstream from the TAA stop translation codon and probably serves as a rho factor-independent transcription terminator (60). The calculated free energy for the stem-loop structure is −18.5 kcal/mol, a value which corresponds to rho factor-independent transcriptional terminators of bacterial genes (67).

The overall G + C content of the coding region of the 21-kDa protein is 60.9%, a value which agrees with the reported G + C content of 61.6 to 62.6% for the *B. avium* genome (44). However, there are regions of higher G + C frequency within the gene encoding the 21-kDa protein; for example, between nucleotides 377 and 399 the G + C content is 86.9%.

Comparison of the deduced amino acid sequence of the 21-kDa protein gene with those of other proteins. The amino acid sequence of the *B. avium* 21-kDa protein was compared with those of proteins deduced from DNA data bases and with the amino acid sequences in protein data bases. As shown in Fig. 5, the 21-kDa protein has extensive homology to the carboxy termini of outer membrane proteins of enteric and nonenteric bacteria. The homologous proteins and their percentages of identity with the *B. avium* 21-kDa protein are as follows: *Neisseria gonorrhoeae* outer membrane protein P III precursor, 33.3 (32); *Enterobacter aerogenes* OmpA precursor, 39.4 (10); *S. typhimurium* OmpA precursor, 39.8 (24); *E. coli* peptidoglycan-associated lipoprotein (pal) precursor, 34 (13); *E. coli* OmpA precursor, 39 (6, 14, 52); *Shigella dysenteriae* OmpA precursor, 39 (9); *Haemophilus influenzae* outer membrane protein P6 precursor, 36.1 (56); *Pseudomonas aeruginosa* porin protein F precursor, 38.8 (22); and *Serratia marcescens* OmpA precursor, 35.6 (11).

Homology of the *B. avium ompA* gene to DNA sequences in other *Bordetella* species. The *B. avium ompA* gene was hybridized with *Eco*RI-digested, chromosomal DNA from *B. avium* and *B. avium*-like bacteria and *B. pertussis* and *B. bronchiseptica* in order to determine whether other *Bordetella* species contain homologous DNA sequences. A DNA fragment internal to the *B. avium ompA* gene hybridized with 6.0- and 3.2-kb DNA fragments from *B. avium* and *B. avium*-like chromosomal DNA, respectively (Fig. 6). *B. pertussis* and *B. bronchiseptica* chromosomal DNA contained 4.1-kb fragments which hybridized with the *B. avium ompA* gene under stringent hybridization conditions.

Production of the *B. avium* 21-kDa outer membrane protein (OmpA) in *E. coli* and *S. typhimurium*. The gene encoding the 21-kDa outer membrane protein of *B. avium* 197 was introduced into *E. coli* χ6097 and *S. typhimurium* χ3987 in order to construct a live avirulent vaccine for oral immunization to protect birds against *B. avium*. Since transcription of *E. coli ompA* is normally stimulated by cyclic AMP and *E. coli cya* and *crp* mutants express *ompA* at a reduced level compared with that of wild-type *E. coli* (30, 51), it was possible that *B. avium ompA* might be similarly regulated. Examination of the upstream region of *B. avium ompA* (Fig. 4) indicated that the promoter region of *B. avium ompA* lacks the cyclic AMP receptor protein-binding sequence (TCACACTT) common to *E. coli ompA* (16). Therefore, it was expected that

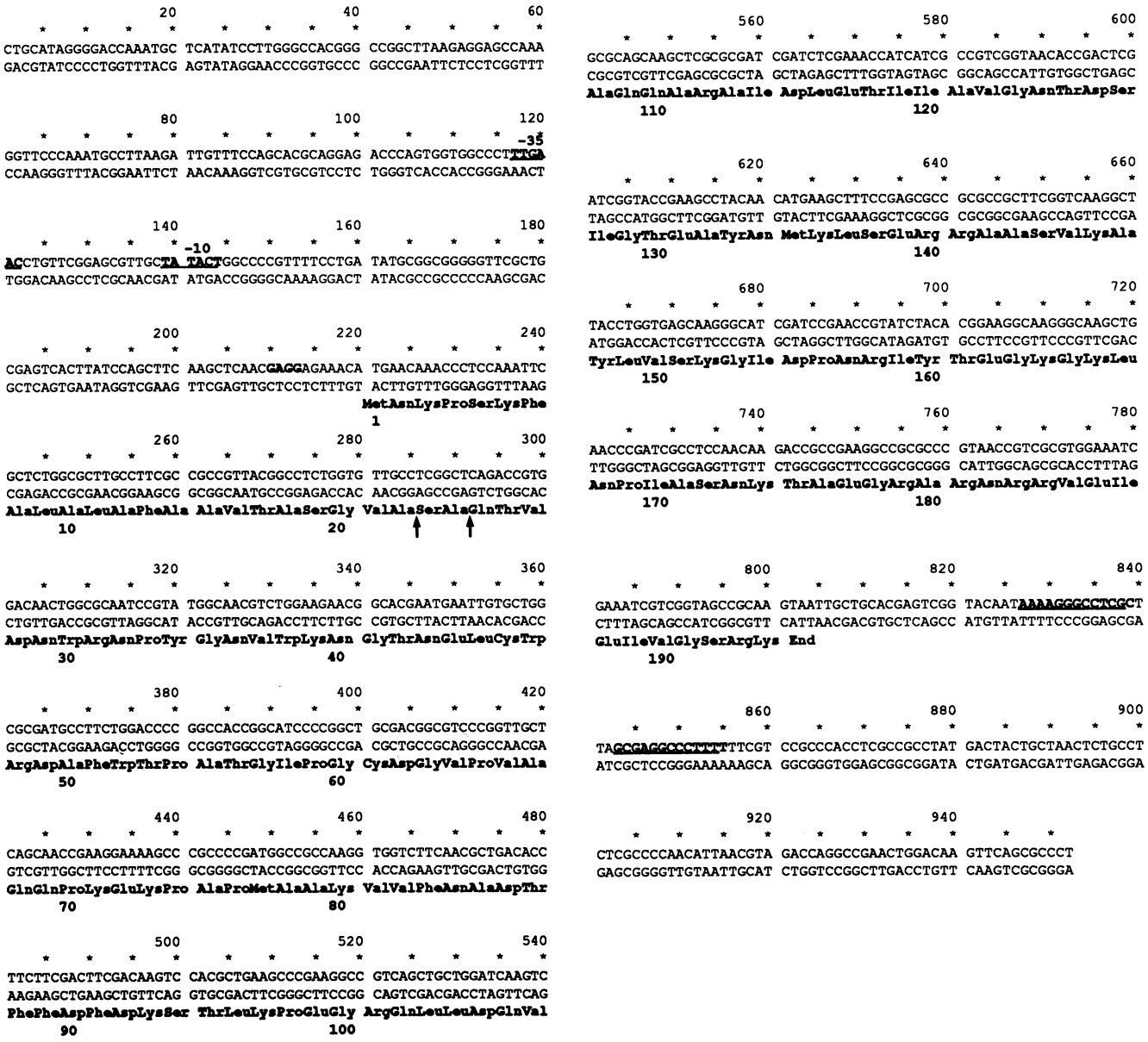

FIG. 4. Nucleotide sequence of the gene encoding the *B. avium* 21-kDa protein and the deduced amino acid sequence of the protein. A putative promoter sequence (−35 and −10 regions) is shown. A potential ribosome-binding site is in boldface print. Arrows designate possible cleavage sites for signal peptidase. A stem-loop region which probably serves as a rho factor-independent transcription terminator is in boldface and is underlined.

expression of *B. avium* 197 *ompA* would not be affected by the lack of *cya* or *crp* in the vaccine strains. *E. coli* χ6097(pYA2336) and *S. typhimurium* χ3987(pYA2336) were examined to determine whether these constructs produced an adequate level of the 21-kDa outer membrane protein for use as an immunogen. Western blot analysis of the proteins produced by *E. coli* LE392(pYA2320), *E. coli* χ6097 (pYA2336), and *S. typhimurium* χ3987(pYA2336) revealed that the *B. avium* OmpA protein is produced at approximately equivalent levels in *B. avium* 197, *E. coli* LE392, *E. coli* χ6097, and *S. typhimurium* χ3987 (Fig. 7). However, while there was one immunoreactive protein band which corresponded to the 21-kDa protein in *B. avium* 197 cell lysates, there were four immunoreactive protein bands produced by *E. coli* LE392 and five immunoreactive protein bands in *E. coli* χ6097 and *S. typhimurium* χ3987 cell lysates. Similar immunoreactive band patterns have been reported in cell lysates of *E. coli* which produce *N. gonorrhoeae* OmpA (32) or overproduce *E. coli* OmpA (26). At least for *E. coli* OmpA, the immunoreactive protein bands have been identified as (i) OmpA, which contains the signal peptide and is located in the cytoplasm or is associated with the cytoplasmic membrane (pro-OmpA); (ii) immature, processed OmpA without the signal peptide, which is found in the periplasm or attached to the inner face of the outer membrane (imp-OmpA); and (iii) mature OmpA (26). An additional protein band, which is thought to be a nascent intermediate protein which arises because of translational pausing and truncation

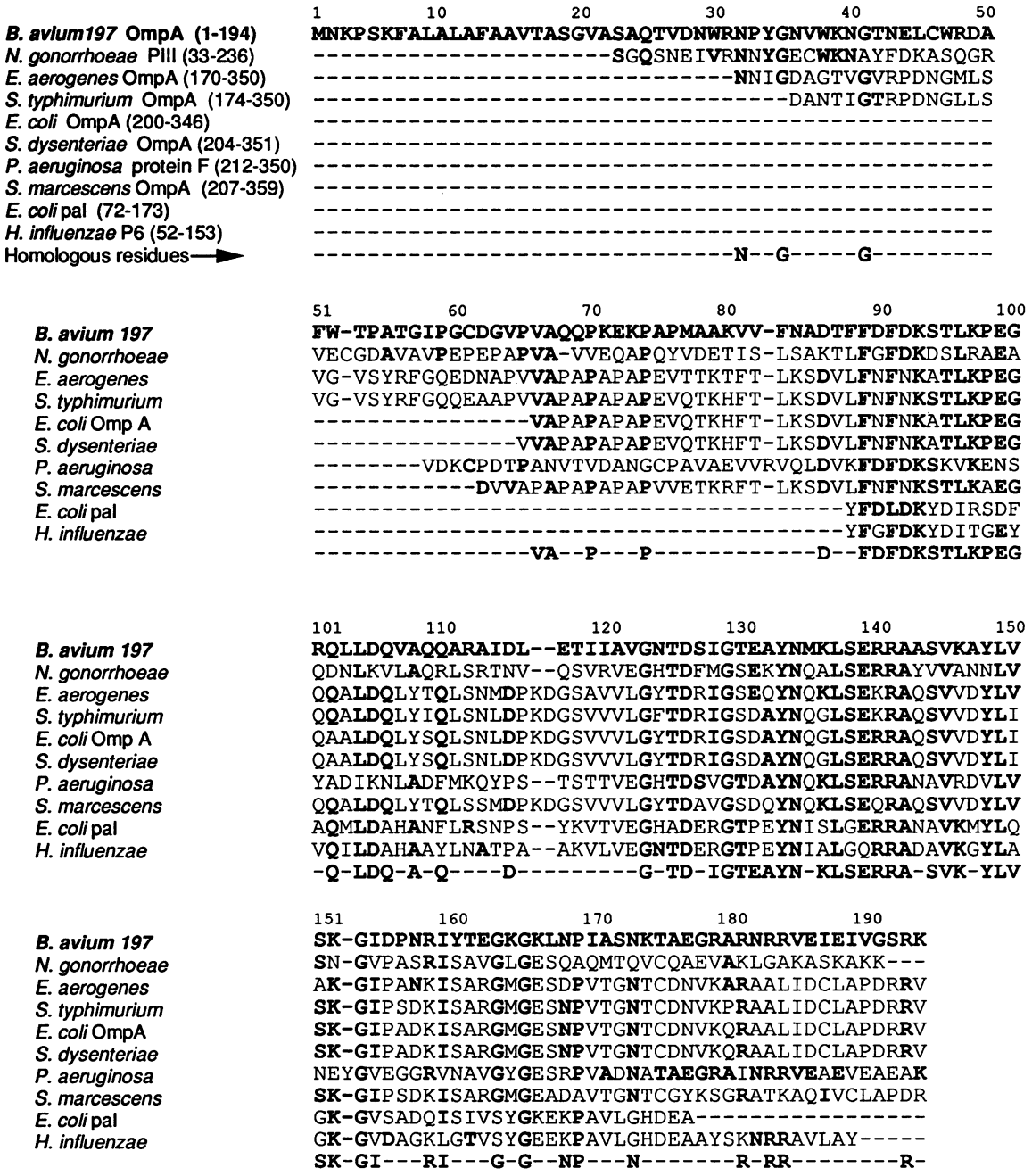

FIG. 5. Comparison of the primary structure of the *B. avium* 21-kDa protein (OmpA) with those of OmpA proteins from enteric and nonenteric bacteria. The top line indicates the amino acid sequence of *B. avium* OmpA. The amino acids of the *B. avium* OmpA protein are numbered from the N terminus of the precursor (unprocessed) protein. The regions of amino acid homology between the *B. avium* OmpA and other OmpA proteins are listed in parentheses next to the respective bacteria. Amino acids of the *B. avium* OmpA precursor protein identical to those of other OmpA precursor proteins are designated in boldface. Dashes indicate nonmatches between the amino acid sequences. The bottom line indicates amino acids of *B. avium* OmpA identical to those of two or more OmpA proteins from other bacteria.

of OmpA, has been seen in pulse-chase experiments with *E. coli* cells expressing *ompA*.

Localization of the *B. avium* 21-kDa outer membrane protein in *S. typhimurium* χ3987(pYA2336). Since the *B. avium* 21-kDa outer membrane protein was homologous to a number of outer membrane proteins from other bacteria and since the gene encoding the 21-kDa protein was expressed at a high level in *S. typhimurium* χ3987(pYA2336), we proceeded to determine whether the 21-kDa *B. avium* protein remained in the cytoplasm, periplasm, or inner membrane or was exported to the outer membrane of *S. typhimurium* χ3987 (pYA2336). Fractionation of *S. typhimurium* χ3987(pYA2336) and Western blot analysis of the fractions revealed that the 21-kDa protein was localized in the inner and outer membranes of *S. typhimurium* χ3987(pYA2336), while a negligible amount of the protein was present in the soluble fractions,

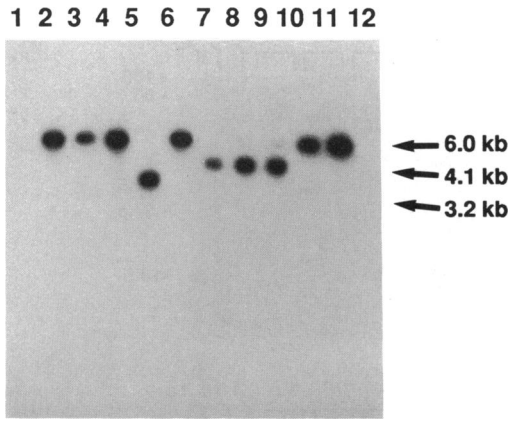

FIG. 6. Southern hybridization of *B. avium ompA* to *Bordetella* chromosomal DNA. *Eco*RI-digested chromosomal DNA was hybridized with a 487-bp DNA fragment internal to the *B. avium ompA* gene. Lanes: 2, *B. avium* 4671; 3, *B. avium* 838; 4, *B. avium* 27/83-T1; 5, *B. avium*-like 031; 6, *B. avium* 002 isolate B; 7, *B. bronchiseptica* 469, 8; *B. pertussis* BB114; 9, *B. pertussis* Tohama I; 10, *B. avium* 105; 11, *B. avium* 197; 1 and 12, lambda phage DNA molecular weight markers.

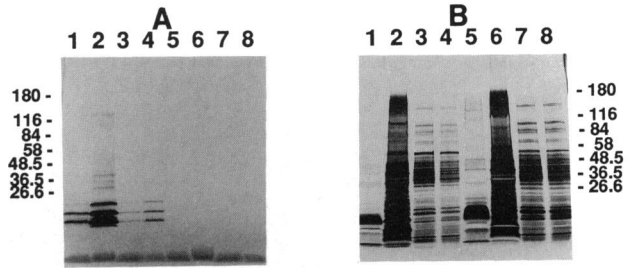

FIG. 8. Localization of the *B. avium* 21-kDa protein in *S. typhimurium* χ3987 cells expressing *B. avium ompA*. (A) Western blot of fractionated proteins reacted with antibody against *B. avium* 197 outer membrane proteins. (B) SDS-PAGE (4 to 20% polyacrylamide gel) and Coomassie blue staining of fractionated proteins. Lanes: 1 and 5, outer membrane fractions of *S. typhimurium* χ3987(pYA2336) and χ3987(pYA292), respectively; 2 and 6, inner membrane fractions of *S. typhimurium* χ3987(pYA2336) and χ3987(pYA292), respectively; 3 and 7, soluble fractions of *S. typhimurium* χ3987(pYA2336) and χ3987(pYA292), respectively; 4 and 8, crude cell lysates of *S. typhimurium* pYA2336 and pYA292, respectively.

which consisted of the periplasmic and cytoplasmic contents (Fig. 8). The outer membrane fraction contained two immunoreactive protein bands which may represent the 21-kDa protein and nascent, truncated 21-kDa protein, while the inner membrane fraction contained five immunoreactive protein bands. Although the amino acid sequence of the 21-kDa *B. avium* protein was highly homologous to the C-terminal amino acids of *S. typhimurium* OmpA, the polyclonal rabbit sera against *B. avium* 197 outer membrane proteins failed to react with *S. typhimurium* χ3987 proteins (Fig. 8A, lanes 5 through 8).

Bird protection studies. Since turkeys naturally become resistant to colonization with *B. avium* 197 at approximately 11 to 14 days of age, the immunization protocol consisted of a single dose of the *S. typhimurium* vaccine strain at 1 day of age followed by challenge with *B. avium* 197 at 9 days postimmunization. Antibodies against the *B. avium* 21-kDa protein were not detectable by Western immunoblot assay in sera from birds immunized with either *S. typhimurium* χ3987(pYA2336) or *S. typhimurium* χ3987(pYA292) and challenged with *B. avium* 197 (Fig. 9). At days 7, 10, and 18 postchallenge, sera from *B. avium*-challenged birds (Fig. 9A) contained antibodies against other *B. avium* 197 proteins rather than the 21-kDa protein. There was considerable variation in the serum antibody response against *S. typhimurium* vaccine constructs in individual birds (Fig. 9B); however, antibodies against *S. typhimurium* proteins were present in all immunized birds by day 19 postimmunization.

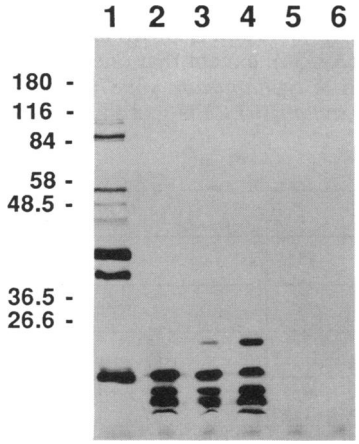

FIG. 7. Western blot comparing the expression of the *B. avium ompA* gene in *B. avium* 197 with expression in *E. coli* LE392, *E. coli* χ6097, and *S. typhimurium* χ3987. Lanes: 1, *B. avium* 197; 2, *E. coli* LE392(pYA2320); 3, *E. coli* χ6097(pYA2336); 4, *S. typhimurium* χ3987(pYA2336); 5, *E. coli* χ6097(pYA292); 6, *S. typhimurium* χ3987(pYA292). Proteins were separated by electrophoresis through an SDS–10% polyacrylamide gel, transferred to a nitrocellulose filter, and reacted with antibody against *B. avium* 197 outer membrane proteins.

Turkeys orally inoculated with *S. typhimurium* χ3987 containing either pYA292 or pYA2336 were found to contain less than 1×10^2 CFU of *S. typhimurium* in the small intestine (both wall and contents) at day 13 postimmunization (data not shown). The average titers of *S. typhimurium* χ3987(pYA292) in the bursa and spleen were 2.8×10^4 and 1.9×10^3 CFU, respectively, at day 13 postimmunization, which slowly declined to less than 1×10^2 CFU by day 27 postimmunization (Fig. 10). In contrast, the average titers of *S. typhimurium* χ3987(pYA2336) in the bursa and spleen were 1.6×10^3 and 6.8×10^4 CFU, respectively, at day 13 postimmunization, which quickly declined to less than 1×10^2 CFU by day 16 postimmunization (Fig. 10). Replica plating of χ3987(pYA292) and χ3987(pYA2336) CFU recovered from various organs at later time points to MacConkey agar containing 1% maltose without diaminopimelic acid revealed that all CFU still contained the plasmids.

Enumeration of *B. avium* 197 in the trachea at day 4 postchallenge (equivalent to day 13 postimmunization) indicated an average level of approximately 3.3×10^7 CFU/g of trachea in nonimmunized turkeys, while turkeys immunized with either *S. typhimurium* χ3987(pYA292) or *S. typhimurium* χ3987(pYA2336) were colonized with 3.2×10^7 and 1.1×10^8 CFU of *B. avium* 197, respectively, per g of trachea. The quantity of *B. avium* 197 in the trachea remained at approximately the same level throughout the experiment and

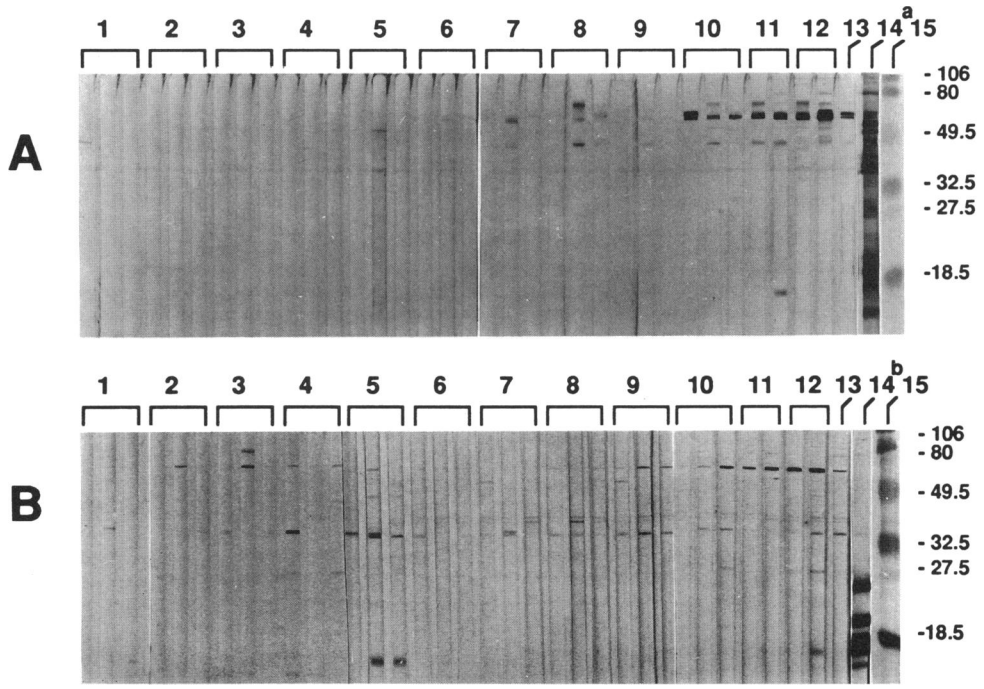

FIG. 9. Western immunoblots of *B. avium* 197 and *S. typhimurium* χ3987(pYA2336) proteins reacted with sera from turkeys immunized at 1 day of age with *Salmonella* vaccine strains and challenged with *B. avium* 197 9 days postimmunization. Each lane in the Western blot indicates the reactivity of serum from a single bird with either *B. avium* 197 (A) or *S. typhimurium* χ3987(pYA2336) (B) proteins. Section 1 shows three lanes which were reacted with sera from nonimmunized, nonchallenged birds. Sections 2, 3, and 4 each show three lanes which were reacted with sera from birds necropsied at 4 days postchallenge which were nonimmunized, immunized with *S. typhimurium* χ3987(pYA292), and immunized with *S. typhimurium* χ3987(pYA2336), respectively. Sections 5, 6, and 7 each show three lanes which were reacted with sera from birds necropsied at 7 days postchallenge which were nonimmunized, immunized with *S. typhimurium* χ3987(pYA292), and immunized with *S. typhimurium* χ3987(pYA2336), respectively. Sections 8, 9, and 10 show three lanes which were reacted with sera from birds necropsied at 10 days postchallenge which were nonimmunized, immunized with *S. typhimurium* χ3987(pYA292), and immunized with *S. typhimurium* χ3987(pYA2336), respectively. Sections 11 and 12 show two lanes which were reacted with sera from birds necropsied at 18 days postchallenge which were nonimmunized and immunized with *S. typhimurium* χ3987(pYA292), respectively. Lane 13 was reacted with serum from one bird necropsied at 18 days postchallenge which was immunized with *S. typhimurium* χ3987(pYA2336). Lanes 14^a and 14^b were reacted with rabbit antibody against *B. avium* 197 outer membrane proteins. Lane 15 shows prestained molecular weight standards.

did not differ significantly between the immunized and nonimmunized birds.

The titer of *B. avium* 197 in the thymus at days 4 and 7 postchallenge was less than 1×10^2 CFU/g for birds immunized with *S. typhimurium* χ3987(pYA292) or *S. typhimurium* χ3987(pYA2336), except that one bird which had been inoculated with *S. typhimurium* χ3987(pYA2336) had a titer of 3.7×10^5 *B. avium* 197 CFU/g of thymus. Nonimmunized

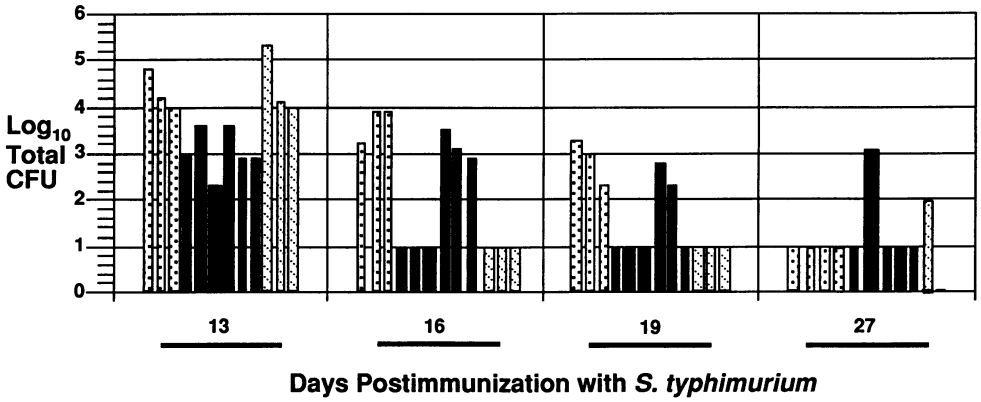

FIG. 10. Recovery of *S. typhimurium* χ3987(pYA292) from the bursa (□) or spleen (■) and recovery of *S. typhimurium* χ3987(pYA2336) from the bursa (▩) or spleen (□) at specified times after peroral inoculation of 2.9×10^9 CFU of χ3987(pYA292) or 1.5×10^9 CFU of χ3987(pYA2336). Each bar represents $\log_{10}$ CFU recovered from an individual bird.

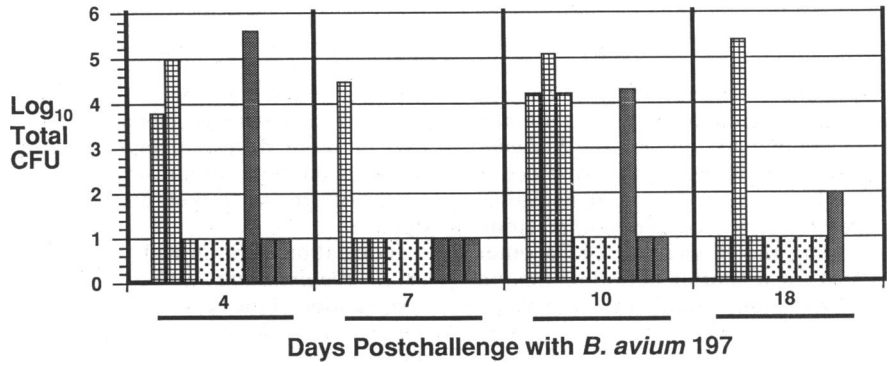

FIG. 11. Recovery of *B. avium* 197 at the specified times from the thymuses of birds orally inoculated with *S. typhimurium* χ3987(pYA292) (▨), *S. typhimurium* χ3987(pYA2336) (▩), or buffered saline gelatin (▦) and challenged with *B. avium* 197. Each bar represents $\log_{10}$ CFU recovered from an individual bird.

turkeys were found to contain an average of 3.9×10^4 CFU of *B. avium* 197 per g of thymus at day 4 postchallenge, and *B. avium* 197 was present in the thymus throughout the experiment (Fig. 11).

DISCUSSION

The structural and functional roles of individual outer membrane proteins of *B. avium* are largely unknown. Therefore, the purpose of this study was to initiate genetic characterization of the outer membrane proteins of *B. avium* and to obtain information on their roles in the structural integrity of the bacterial cell and in disease. A gene library of *B. avium* DNA was constructed in *E. coli* to allow isolation of *B. avium* genes encoding outer membrane proteins. Five *E. coli* clones were identified which contained recombinant cosmids encoding *B. avium* outer membrane proteins with molecular masses of 21, 38, 40, 43, and 48 kDa. Since previous studies indicated that sera and tracheal washings from *B. avium*-infected turkeys showed the greatest increase in antibodies against a 21-kDa outer membrane protein of *B. avium* over the course of infection (38) and since the *E. coli* clone encoding the 21-kDa outer membrane protein reacted with turkey convalescent-phase sera, we focused our studies on the gene encoding the 21-kDa protein.

Nucleotide sequence analysis of the 21-kDa protein gene indicated that it encodes 194 amino acids which result in a protein with a molecular mass of 21,113 Da. The 21-kDa protein contains extensive homology to OmpA proteins from numerous bacteria, and on the basis of this homology, we propose the designation of *ompA* for the gene encoding the *B. avium* 21-kDa protein. The OmpA protein of *E. coli* has several functions, including (i) maintenance of cell structure and integrity, (ii) bacteriophage receptor, (iii) involvement in colicin uptake, and (iv) mediator in F-dependent conjugation (for a review, see reference 45). The functional domains of *E. coli* OmpA have been well-characterized and a model for the arrangement of OmpA in the outer membrane has been constructed (12, 14, 25, 50). *E. coli* OmpA is composed of a transmembrane portion from amino acid residues 1 to 177 (12, 14), an Ala-Pro repeat region from residues 201 to 208, and a periplasmic portion from residue 209 to the end of the protein. Regions around amino acid residues 25, 70, 110, and 154 are exposed on the cell surface (50) and therefore are necessarily involved in colicin uptake, bacteriophage infection, and conjugation. *B. avium* OmpA lacks homology to the amino terminus of *E. coli* OmpA; therefore, it is difficult to assign similar functions to the amino terminus of *B. avium* OmpA. *B. avium* OmpA also lacks an Ala-Pro repeat but contains a proline-rich region from residues 54 to 76 (in the *B. avium* OmpA precursor) which corresponds to the Ala-Pro repeat region of *E. coli* OmpA. The region of homology between the *B. avium* OmpA and the OmpA proteins from other bacteria is limited to the carboxy terminus of the protein, a region which encompasses amino acids 86 to 194 of the *B. avium* OmpA precursor. If the proline-rich region of *B. avium* OmpA serves to separate the periplasmic and transmembrane portions of the protein, as occurs in *E. coli* OmpA, then a very limited area of approximately 30 to 40 amino acids of the *B. avium* OmpA is transmembrane and/or surface located. However, examination of surface probability and antigenic-index plots of *B. avium* OmpA (data not shown) suggests that the N-terminal amino acids are exposed on the surface of *B. avium*. Additional experiments are necessary to confirm whether there is a topological analogy between *B. avium* OmpA and *E. coli* OmpA transmembrane and the periplasmic moieties of the protein.

The *B. avium* OmpA protein lacks RGD (Arg-Gly-Asp) sequences, amino acid sequences which are commonly involved in adhesion to mammalian cells (58). However, since the titer of antibody to a 21-kDa outer membrane protein increased during infection (38), the possibility of *B. avium* OmpA as an immunogen against bordetellosis in birds needs to be explored further. Outer membrane proteins which are homologous to the *B. avium* OmpA protein have been implicated as important immunogens against human diseases. *P. aeruginosa* porin protein F and *H. influenzae* protein P6 are protective in the burned-mouse and infant-rat models, respectively, and both proteins are currently being considered as vaccine candidates (34, 49, 53). In contrast, *N. gonorrhoeae* protein P III induces both protective bactericidal antibodies and harmful blocking antibodies which interfere with the complement-activated killing of the gonococci by human immune sera (2, 59). By analogy, the *B. avium* OmpA protein may be a potential vaccine candidate or may induce blocking antibodies and exacerbate disease. Alternatively, *B. avium* OmpA may simply be a structural protein which protects the integrity of the bacterium but plays no role in disease.

To determine whether *B. avium* OmpA could induce protective immunity when delivered to the immune systems of birds via an oral vaccine delivery system, avirulent *S. typhimurium* strains were constructed which expressed *B. avium ompA*. The *B. avium ompA* gene was subcloned into

the Asd$^+$ vector pYA292, and the construct was introduced into an avirulent Δ*cya* Δ*crp* Δ*asd* *S. typhimurium* χ3987 and used to orally immunize the birds. The results of these immunization experiments must be viewed with caution since these studies were performed once with a limited number of birds. In these preliminary studies, the *S. typhimurium* vaccine strain was present in the bursa and spleen at 13 days postimmunization but was recovered at negligible levels from the small intestine. The *S. typhimurium* strain expressing the gene encoding the *B. avium* 21-kDa outer membrane protein survived for a shorter period of time in vivo than the *S. typhimurium* strain containing the plasmid vector alone, suggesting that expression of the *B. avium* 21-kDa outer membrane protein might be deleterious to prolonged survival of *S. typhimurium* χ3987 in vivo.

Challenge of nonimmunized turkeys and turkeys immunized with *S. typhimurium* expressing the gene encoding the *B. avium* 21-kDa outer membrane protein with wild-type *B. avium* 197 resulted in colonization of the trachea and the thymus by *B. avium*. Tracheal colonization by *B. avium* 197 was not surprising, since serum antibody against the 21-kDa protein was not induced by the immunization protocol and therefore could not transudate from the systemic circulation and block adherence of the bacterium. In this study, only serum antibody was tested for antibody against the 21-kDa protein and the birds were not examined for a mucosal immune response, so it is difficult to assess whether or not the immunization protocol stimulated a mucosal immune response against the 21-kDa protein.

There are several possible explanations for the lack of a serum immune response against the 21-kDa protein in birds immunized with *S. typhimurium* expressing the gene encoding the *B. avium* protein. First, the gene encoding the 21-kDa protein may be unstable in vivo, and consequently, the 21-kDa protein may not be available as an immunogen in vivo. Second, the 21-kDa *B. avium* 197 outer membrane protein may not be immunogenic when delivered via the *S. typhimurium* oral vaccine system. Although it was not tested in this study, parenteral injection of purified *B. avium* OmpA into birds might induce serum antibodies and protect birds from challenge with *B. avium*. This possibility will be addressed in future studies. Third, the high immunizing dose of the *S. typhimurium* vaccine strain and the young age of the birds may result in lymphocyte depletion and/or suppression of the immune response. It has been speculated (36) and recently demonstrated that wild-type *S. typhimurium* can cause lymphocyte depletion and suppression of the immune response in 3- and 7-day-old chickens (37). Although the *S. typhimurium* χ3985 vaccine strain does not induce this immune suppression in young chicks, high doses of the vaccine strain in 1-day-old young turkeys may be responsible for the lack of serum antibodies against the *B. avium* 21-kDa protein. Fourth, a single immunizing dose of the vaccine may not be adequate for induction of a protective immune response. Recent studies have indicated that a single immunizing dose of *S. typhimurium* χ3985 reduces fecal excretion of wild-type *S. typhimurium* in chickens but a double immunization is required to protect chickens from cecal colonization by wild-type *S. typhimurium* (36). As mentioned previously, the early age of infection with *B. avium* precludes the use of booster immunizations to prevent *B. avium* colonization. Fifth, the period of time between oral inoculation with the *S. typhimurium* vaccine strain and challenge with *B. avium* may not be adequate for induction of an immune response against the 21-kDa protein because of the immaturity of the bird immune system. The latter two hypotheses seem plausible, since antibodies against *S. typhimurium* were not consistently present in serum at a detectable level until day 19 postimmunization. Since *B. avium* infection is initiated early in the life of birds, an alternative immunization strategy might include oral immunization, in two or more doses, of layer hens. Passive immunity by maternal antibody would serve to protect chicks until 3 or 4 weeks of age, when their immune systems have fully developed.

Inoculation of birds with *S. typhimurium* χ3987(pYA292) or *S. typhimurium* χ3987(pYA2336) resulted in reduced colonization of the thymus by *B. avium* 197. Furthermore, *B. avium* 197 colonized the turkey tracheas regardless of whether or not the bacterium was present in the thymus. One explanation for this result is that immunization with *S. typhimurium* χ3987 may stimulate a nonspecific T-cell response which limits *B. avium* colonization in the thymus but has no effect on tracheal colonization.

The absence of serum antibody against the 21-kDa protein in immunized birds makes it difficult to determine whether the 21-kDa protein is an antigen protective against bordetellosis. Further bird infectivity studies utilizing *B. avium* mutants lacking the 21-kDa protein and studies on parenteral immunization with the purified protein will need to be performed to determine the role of the 21-kDa protein in bordetellosis.

ACKNOWLEDGMENTS

We thank Vincent Collins for his scientific discussions, suggestions, and technical assistance in enumeration of *B. avium* in the thymus in bird infectivity studies. In addition, we thank H. A. Berkhoff, W. E. Goldman, M. S. Hofstad, A. Honeyman, Y. M. Saif, D. G. Simmons, J. K. Skeeles, and E. Tuomanen for providing bacterial strains. We also thank J. Thompson, J. P. London, and J. Manch-Citron for critical review of the manuscript and for suggestions.

This work was partially supported by National Research Service award 1-F32-AI-07628, United States Department of Agriculture grant 86-CRSR-2-2854, and Public Health Service grant AI-28487 from the National Institutes of Health.

REFERENCES

1. **Akeila, M. A., and Y. M. Saif.** 1988. Protection of turkey poults from *Bordetella avium* infection and disease by pili and bacterins. Avian Dis. **32**:641–649.
2. **Arminjon, F., M. Cadoz, S. A. Morse, J. P. Rock, and S. K. Sarafian.** 1987. Bactericidal and opsonic activities of sera from individuals immunized with a gonococcal protein I vaccine, E-92, p. 118. Abstr. Annu. Meet. Am. Soc. Microbiol. 1987. American Society for Microbiology, Washington, D.C.
3. **Arp, L. H., and N. F. Cheville.** 1984. Tracheal lesions in young turkeys infected with *Bordetella avium*. Am. J. Vet. Res. **45**:2196–2200.
4. **Arp, L. H., and J. A. Fagerland.** 1987. Ultrastructural pathology of *Bordetella avium* infection in turkeys. Vet. Pathol. **24**:411–418.
5. **Arp, L. H., R. D. Leyh, and R. W. Griffith.** 1988. Adherence of *Bordetella avium* to tracheal mucosa of turkeys: correlation with hemagglutination. Am. J. Vet. Res. **49**:693–696.
6. **Beck, E., and E. Bremer.** 1980. Nucleotide sequence of the gene *ompA* coding the outer membrane protein II* of *Escherichia coli*. Nucleic Acids Res. **8**:3011–3024.
7. **Berkhoff, H. A., F. M. McCorkle, Jr., and T. T. Brown.** 1983. Pathogenicity of various isolates of *Alcaligenes faecalis* for broilers. Avian Dis. **27**:707–713.
8. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
9. **Braun, G., and S. T. Cole.** 1982. The nucleotide sequence

coding for major outer membrane protein OmpA of *Shigella dysenteriae*. Nucleic Acids Res. **10**:2367–2378.
10. **Braun, G., and S. T. Cole.** 1983. Molecular characterization of the gene coding for major outer membrane protein OmpA from *Enterobacter aerogenes*. Eur. J. Biochem. **137**:495–500.
11. **Braun, G., and S. T. Cole.** 1984. DNA sequence analysis of the *Serratia marcescens ompA* gene: implications for the organisation of an enterobacterial outer membrane protein. Mol. Gen. Genet. **195**:321–328.
12. **Bremer, E., S. T. Cole, I. Hindennach, U. Henning, E. Beck, C. Kurz, and H. Schaller.** 1982. Export of a protein into the outer membrane of *Escherichia coli* K12. Eur. J. Biochem. **122**:223–231.
13. **Chen, R., and U. Henning.** 1987. Nucleotide sequence of the gene for the peptidoglycan-associated lipoprotein of *Escherichia coli* K12. Eur. J. Biochem. **163**:73–77.
14. **Chen, R., W. Schmidmayr, C. Kramer, U. Chen-Schmeisser, and U. Henning.** 1980. Primary structure of major outer membrane protein II* (OmpA protein) of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **77**:4592–4596.
15. **Close, T. J., D. Zaitlin, and C. I. Kado.** 1984. Design and development of amplifiable broad-host-range cloning vectors: analysis of the *vir* region of *Agrobacterium tumefaciens* plasmid pTiC58. Plasmid **12**:111–118.
16. **Cole, S. T., E. Bremer, I. Hindennach, and U. Henning.** 1982. Characterization of the promoters for the *ompA* gene which encodes a major outer membrane protein of *Escherichia coli*. Mol. Gen. Genet. **188**:472–479.
17. **Collins, V., and R. Curtiss III.** 1992. Unpublished data.
18. **Curtiss, R., III, and S. M. Kelly.** 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. **55**:3035–3043.
19. **Curtiss, R., III, S. B. Porter, M. Munson, S. A. Tinge, J. O. Hassan, C. Gentry-Weeks, and S. M. Kelly.** 1991. Nonrecombinant and recombinant avirulent *Salmonella* live vaccines for poultry, p. 169–198. *In* L. C. Blankenship, J. S. Bailey, N. A. Cox, N. J. Stern, and R. J. Meinersmann (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, New York.
20. **Darzins, A., and A. M. Chakrabarty.** 1984. Cloning of genes controlling alginate biosynthesis from a mucoid cystic fibrosis isolate of *Pseudomonas aeruginosa*. J. Bacteriol. **159**:9–18.
21. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Duchene, M., A. Schweizer, F. Lottspeich, G. Krauss, M. Marget, K. Vogel, B.-U. von Specht, and H. Domdey.** 1988. Sequence and transcriptional start site of the *Pseudomonas aeruginosa* outer membrane porin protein F gene. J. Bacteriol. **170**:155–162.
23. **Freeman, D., and G. Luginbuhl (North Carolina State University).** 1991. Personal communication.
24. **Freudl, R., and S. T. Cole.** 1983. Cloning and molecular characterization of the *ompA* gene from *Salmonella typhimurium*. Eur. J. Biochem. **134**:497–502.
25. **Freudl, R., M. Klose, and U. Henning.** 1990. Export and sorting of the *Escherichia coli* outer membrane protein OmpA. J. Bioenerg. Biomembr. **22**:441–449.
26. **Freudl, R., H. Schwarz, Y.-D. Stierhof, K. Gamon, I. Hindennach, and U. Henning.** 1986. An outer membrane protein (OmpA) of *Escherichia coli* K-12 undergoes a conformational change during export. J. Biol. Chem. **261**:11355–11361.
27. **Galan, J. E., K. Nakayama, and R. Curtiss III.** 1990. Cloning and characterization of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene **94**:29–35.
28. **Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss III.** 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of *Bordetella avium*. Infect. Immun. **56**:1698–1707.
29. **Gentry-Weeks, C. R., D. L. Provence, J. M. Keith, and R. Curtiss III.** 1991. Isolation and characterization of *Bordetella avium* phase variants. Infect. Immun. **59**:4026–4033.
30. **Gibert, I., and J. Barbe.** 1990. Cyclic AMP stimulates transcription of the structural gene of the outer-membrane protein OmpA of *Escherichia coli*. FEMS Microbiol. Lett. **68**:307–312.
31. **Gold, L., D. Pribnow, T. Schneider, S. Shinedling, B. S. Singer, and G. Stormo.** 1981. Translational initiation in prokaryotes. Annu. Rev. Microbiol. **35**:365–403.
32. **Gotschlich, E. C., M. Seiff, and M. S. Blake.** 1987. The DNA sequence of the structural gene of gonococcal protein III and the flanking region containing a repetitive sequence. J. Exp. Med. **165**:471–482.
33. **Gray, J. G., J. F. Roberts, R. C. Dillman, and D. G. Simmons.** 1983. Pathogenesis of change in the upper respiratory tracts of turkeys experimentally infected with an *Alcaligenes faecalis* isolate. Infect. Immun. **42**:350–355.
34. **Green, B. A., B. J. Metcalf, T. Quinn-Dey, D. H. Kirkley, S. A. Quataert, and R. A. Deich.** 1990. A recombinant non-fatty acylated form of the Hi-PAL (P6) protein of *Haemophilus influenzae* elicits biologically active antibody against both nontypeable and type b *H. influenzae*. Infect. Immun. **58**:3272–3278.
35. **Hanna, Z., C. Fregeau, G. Prefontaine, and R. Brousseau.** 1984. Construction of a family of universal expression plasmid vectors. Gene **30**:247–250.
36. **Hassan, J. O., and R. Curtiss III.** 1990. Control of colonization by virulent *Salmonella typhimurium* by oral immunization of chickens with avirulent Δ*cya* Δ*crp S. typhimurium*. Res. Microbiol. **41**:839–850.
37. **Hassan, J. O., and R. Curtiss III.** 1992. Unpublished data.
38. **Hellwig, D. H., and L. H. Arp.** 1990. Identification of *Bordetella avium* antigens recognized after experimental inoculation in turkeys. Am. J. Vet. Res. **51**:1188–1191.
39. **Hellwig, D. H., L. H. Arp, and J. A. Fagerland.** 1988. A comparison of outer membrane proteins and surface characteristics of adhesive and non-adhesive phenotypes of *Bordetella avium*. Avian Dis. **32**:787–792.
40. **Hinz, K.-H., G. Glunder, and H. Luders.** 1978. Acute respiratory disease in turkey poults caused by *Bordetella bronchiseptica*-like bacteria. Vet. Rec. **103**:262–263.
41. **Honeyman, A. (Washington University).** 1991. Personal communication.
42. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
43. **Jackwood, M. W., and Y. M. Saif.** 1987. Pili of *Bordetella avium*: expression, characterization, and role in in vitro adherence. Avian Dis. **31**:277–286.
44. **Kersters, K., K.-H. Hinz, A. Hertle, P. Segers, A. Lievens, O. Siegmann, and J. De Ley.** 1984. *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. Int. J. Syst. Bacteriol. **34**:56–70.
45. **Lugtenberg, B., and L. van Alphen.** 1983. Molecular architecture and functioning of the outer membrane of *Escherichia coli* and other gram-negative bacteria. Biochim. Biophys. Acta **737**:51–115.
46. **Manclark, C. R., and J. L. Cowell.** 1984. Pertussis, p. 69–106. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., New York.
47. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Marshall, D. R., D. G. Simmons, and J. G. Gray.** 1985. An *Alcaligenes faecalis* isolate from turkeys: pathogenicity in selected avian and mammalian species. Am. J. Vet. Res. **46**:1181–1184.
49. **Matthews-Greer, J. M., and H. E. Gilleland, Jr.** 1987. Outer membrane protein F (porin) preparation of *Pseudomonas aeruginosa* as a protective vaccine against heterologous immunotype strains in a burned mouse model. J. Infect. Dis. **155**:1282–1291.
50. **Morona, R., M. Klose, and U. Henning.** 1984. *Escherichia coli* K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins.

J. Bacteriol. **159**:570–578.
51. **Movva, R. N., P. Green, K. Nakamura, and M. Inouye.** 1981. Interaction of cAMP receptor protein with the *ompA* gene, a gene for a major outer membrane protein of *Escherichia coli*. FEBS Lett. **128**:186–190.
52. **Movva, R. N., K. Nakamura, and M. Inouye.** 1980. Gene structure of the OmpA protein, a major surface protein of *Escherichia coli* required for cell-cell interaction. J. Mol. Biol. **143**:317–328.
53. **Munson, R. S., Jr., and D. M. Granoff.** 1985. Purification and partial characterization of outer membrane proteins P5 and P6 from *Haemophilus influenzae* type b. Infect. Immun. **49**:544–549.
54. **Nag, D. K., H. V. Huang, and D. E. Berg.** 1988. Bidirectional chain-termination nucleotide sequencing: transposon Tn*5*seq1 as a mobile source of primer sites. Gene **64**:135–145.
55. **Nakayama, K., S. M. Kelly, and R. Curtiss III.** 1988. Construction of an Asd^+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a *Salmonella* vaccine strain. Bio/Technology **6**:693–697.
56. **Nelson, M. B., M. A. Apicella, T. F. Murphy, H. Vankeulen, L. D. Spotila, and D. Rekosh.** 1988. Cloning and sequencing of *Haemophilus influenzae* outer membrane protein P6. Infect. Immun. **56**:128–134.
57. **Oliver, D.** 1985. Protein secretion in *Escherichia coli*. Annu. Rev. Microbiol. **39**:615–648.
58. **Pierschbacher, M. D., and E. Ruoslahti.** 1984. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. Nature (London) **309**:30–33.
59. **Rice, P. A., H. E. Vayo, M. R. Tam, and M. S. Blake.** 1986. Immunoglobulin G antibodies directed against protein III block killing of serum-resistant *Neisseria gonorrhoeae* by immune serum. J. Exp. Med. **164**:1735–1748.
60. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
61. **Saif, Y. M., P. D. Moorhead, R. N. Dearth, and D. J. Jackwood.** 1980. Observations on *Alcaligenes faecalis* infection in turkeys. Avian Dis. **24**:665–684.
62. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
63. **Sarkar, G., S. Kapelner, and S. S. Sommer.** 1990. Formamide can dramatically improve the specificity of PCR. Nucleic Acids Res. **18**:7465.
64. **Schmieger, H.** 1972. Phage P22-mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119**:75–88.
65. **Simmons, D. G., D. E. Davis, L. P. Rose, J. G. Gray, and G. H. Luginbuhl.** 1981. *Alcaligenes faecalis*-associated respiratory disease of chickens. Avian Dis. **25**:610–613.
66. **Simmons, D. G., J. G. Gray, L. P. Rose, R. C. Dillman, and S. E. Miller.** 1979. Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. Avian Dis. **23**:194–203.
67. **Stroynowski, I., M. Kuroda, and C. Yanofsky.** 1983. Transcription termination in vitro at the tryptophan operon attenuator is controlled by secondary structure in the leader transcript. Proc. Natl. Acad. Sci. USA **80**:2206–2210.
68. **Tanji, Y., J. Gennity, S. Pollitt, and M. Inouye.** 1991. Effect of OmpA signal peptide mutations on OmpA secretion, synthesis, and assembly. J. Bacteriol. **173**:1997–2005.
69. **Tilghman, S. M., D. C. Tiemeier, F. Polsky, M. H. Edgell, J. G. Seidman, A. Leder, L. W. Enquist, B. Norman, and P. Leder.** 1977. Cloning specific segments of the mammalian genome: bacteriophage λ containing mouse globin and surrounding gene sequences. Proc. Natl. Acad. Sci. USA **74**:4406–4410.
70. **Tinge, S. A., and R. Curtiss III.** 1990. Conservation of *Salmonella typhimurium* virulence plasmid maintenance regions among *Salmonella* serovars as a basis for plasmid curing. Infect. Immun. **58**:3084–3092.
71. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
72. **Van Alstine, W. G., and L. H. Arp.** 1988. Histologic evaluation of lung and bronchus-associated lymphoid tissue in young turkeys infected with *Bordetella avium*. Am. J. Vet. Res. **49**:835–839.
73. **von Heijne, G.** 1984. How signal sequences maintain cleavage specificity. J. Mol. Biol. **173**:243–251.