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Direct Expression of *Bordetella pertussis* Filamentous Hemagglutinin in *Escherichia coli* and *Salmonella typhimurium aroA*

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Nonfused (i.e., nonhybrid) filamentous hemagglutinin (FHA) of *Bordetella pertussis* was efficiently expressed in *Escherichia coli* K-12 and *Salmonella typhimurium aroA* at levels higher than those found in wild-type *B. pertussis* when the upstream signals of the gene were replaced and the translation initiation region was engineered to optimize translational efficiency. Inclusion of part of the C-terminal FHA open reading frame, whose translation product does not appear to be part of the major secreted species of FHA, was shown to be important in achieving protein expression in both *E. coli* and *S. typhimurium aroA*; removal of the downstream gene sequence abolished recombinant FHA production. The levels of expression observed varied widely according to the construct and host bacterium used.

Bordetella pertussis is the etiological agent of whooping cough, a major childhood respiratory disease that can develop serious complications and cause death (46). Although whole-cell vaccine preparations routinely administered in combination with diphtheria and tetanus toxoids provide good protection against severe disease, increasing concern regarding the side effects of the vaccines has led to decreased vaccine acceptability, a decrease in the number of vaccinated infants, and a consequent increase in the incidence of whooping cough (24).

B. pertussis produces a number of virulence factors, including pertussis toxin, adenylate cyclase, filamentous hemagglutinin (FHA), fimbriae, and major outer membrane proteins, whose synthesis is positively regulated by the products of the *bvg* locus (14, 36, 48). Some of these determinants are potential antigens for inclusion in new-generation acellular, atoxic, nonreactogenic vaccines (23, 24). One of the most promising components for inclusion in such a vaccine is FHA, which plays a major role in bacterial attachment and subsequent colonization of the epithelial respiratory tract during the early stages of disease (44, 45). FHA may also facilitate the superinfections that commonly complicate this disease because other bacteria may utilize this bridge-acting adhesin (43) and because of the impairment of macrophage responses due to specific interactions mediated by FHA (20). An immune response against FHA might prevent infection and disease by inhibiting colonization (33), inducing opsonization (10), or stimulating cellular responses against the invading bacterium.

Problems in the production of FHA for acellular vaccines include the fermentation of *B. pertussis*, a human pathogen (problems in production safety) and a fastidious microorganism with slow growth rates (long fermentation times, poor yields), and the contamination of FHA preparations with other virulence factors (which may contribute to some of the side effects of vaccination). Here we report the efficient direct expression of nonfused (i.e., nonhybrid) FHA in *Escherichia coli*. Furthermore, we have obtained expression of nonfused FHA in a *Salmonella typhimurium aroA* strain.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains used in this work were as follows. The *B. pertussis* strain used was Tohama serotype 1.2 (34). *E. coli* SURE (Stratagene) *recB recJ sbcC201 uvrC umuC*::Tn*5* (Kn^r) *mcrA mcrB mrr lac* Δ*hsdRMS endA1 gyrA96 thi relA1 supE44* F′ (*proAB lacI*^{q}ZΔM15 Tn*10*) and *E. coli* JM109 *endA1 recA1 hsdR17 supE44 thi gyrA96* Δ(*lac-proAB*) F′ (*traD36 proAB*$^+$ *lacI*^{q}ZΔM15) (49) were used for transformations when recombinant clones were screened for β-galactosidase alpha complementation, whereas *E. coli* CC118 *araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA20 galE galK thi-1 rpsE* (Sp^r) *rpoB argE*(Am) *recA1* Rif^r (17) was used as a recipient strain for other transformations. *E. coli* 537(pCI857^{TS}) (22) was used for the induction of MS2 polymerase fusions. *E. coli* 876 *pep* (R. Brownlie), *E. coli* EC538 (J. E. G. McCarthy), and *E. coli* CAG629 *lon htpR165*::Tn*10* (C. Gross) were used as recipient strains in induction experiments for the expression of FHA. *E. coli* SK5003 F^- *leu thr* Cm^r *rnb-500 pnp-7* (7) was used in experiments to assess mRNA degradation. *E. coli* K38λ(pGP1-2) Km^r (28, 42), BL21 DE3, and BL21 DE3(pLysS) (41) were used for the expression of pT7-7 constructions; *S. typhimurium* SL5283 (B. A. Stocker), a *galE503* derivative of LB5000 *hsdSB121 leu-3121* (31), was used as an intermediate strain in the transfer of plasmids by transformation from *E. coli* to *S. typhimurium* SL3261 *aroA* (12). The plasmids used in this work were pUC18*Not*I (11), pEX31A and pEX31B (40), pJLA503 (37), pT7-7 (S. Tabor), and pRMB2 (2). Plasmid pLG612-1 (I. B. Holland) was used as a source of the 23-kDa COOH-terminal signal sequence of hemolysin A, and plasmid pLG575 (16) was used as a source of the hemolysin B accessory genes.

E. coli and *S. typhimurium* strains were grown in Luria broth or on Luria agar (29). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside medium (29) was used for selection with the β-galactosidase alpha complementation system. *B. pertussis* was grown on Bordet-Gengou agar base (Difco) supplemented with 1% glycerol and 15% (vol/vol) defibrinated horse blood or in SS-X broth (39). The following antibiotics were used for selection: ampicillin (100 μg/ml),

chloramphenicol (50 μg/ml), and tetracycline (50 μg/ml). Broth cultures were aerated by shaking at 200 rpm in a New Brunswick environmental incubator shaker.

DNA manipulations. Plasmids were isolated by alkaline lysis (29). Restriction endonuclease digestion, ligation with T4 DNA ligase, T4 polynucleotide kinase end labeling, dephosphorylation with fetal calf intestinal phosphatase, and transformation were performed as described by Sambrook et al. (29). Enzymes were obtained from Boehringer Mannheim. DNA sequencing was carried out by the chain termination method of Sanger et al. (32). Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA synthesizer in accordance with the manufacturer's instructions.

RNA isolation and primer extension analysis. RNA was isolated as previously described (47). The FHA*a* primer (see below) was labeled at its 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham). Primer extension was carried out as described by de Lorenzo et al. (5). Samples were heated at 80°C for 10 min and electrophoresed as for DNA sequence analysis.

RNA dot blot hybridization. Total RNA (50 μg) was spotted onto nylon membranes (Pall Biodyne type B; 0.45-μm pore size), fixed by being baked at 80°C for 1 h, prehybridized, and hybridized with 5′-end-labeled probes as described by Sambrook et al. (29). Probes complementary to the coding strand were as follows: (i) FHA*a* (3′-AGGCAA TCATTGCGTGCGGGT-5′) complementary to nucleotides 194 to 214 of the published *fhaB* sequence (6, 21); (ii) FHA*b* (3′-ACAGCTCGTTCGTCCCGTTCG-5′) complementary to nucleotides 730 to 750; (iii) FHA*c* (3′-AGGTCGAACCG CGCCGCTCGT-5′) complementary to nucleotides 1228 to 1248; (iv) FHA*d* (3′-GCTTGTCGCTGCGCCTTAACGC-5′) complementary to nucleotides 1695 to 1717; (v) FHA*e* (3′-GCTTGAGCGACGTGCGGTTAG-5′) complementary to nucleotides 2599 to 2619; (vi) FHA*f* (3′-GTGCTAAAGTGC CACGTCC-5′) complementary to nucleotides 4745 to 4764; (vii) FHA*g* (3′-TTCTGCGGAGAAGGCCACTC-5′) complementary to nucleotides 5748 to 5768; (viii) FHA*h* (3′-AGT TCCAGCGCTGGAGCTAC-5′) complementary to nucleotides 7747 to 7767; (ix) jla*501* (3′-AATAGAGACCGCCAC AACTG-5′) complementary to nucleotides 1306 to 1325 upstream of the start of transcription from the p_R and p_L promoters in vector pJLA503 (used as a negative control) (37); and (x) Hly*1* (3′-ATAGAAAGTCCTATACCGGT-5′) complementary to nucleotides 3873 to 3892 of the *E. coli* hemolysin A gene sequence (8). See Fig. 3C for a graphic representation of the probes.

Induction experiments. Overnight cultures were diluted to an A_{590} of 0.4 with Luria broth, grown at 30°C with shaking at 200 rpm to an A_{590} of 0.9, and temperature induced at 42°C or isopropylthio-β-D-galactoside (IPTG) induced by the addition of IPTG to a 1.0 mM final concentration. After induction, 1 ml of the culture was pelleted and resuspended in 0.7% NaCl. For protein secretion screening, 0.9 ml of supernatant fluid was mixed with 100 μl of 100% (wt/vol) trichloroacetic acid, incubated on ice for 2 h, centrifuged at 3,000 × *g* for 15 min, washed with 100% ethanol, and resuspended in 0.7% NaCl. Protein concentrations of samples were determined by the Lowry procedure as modified by Sanderman and Strominger (30). Samples were mixed in a 1:1 ratio with loading buffer (29), and approximately 50 μg of protein was electrophoresed by the procedure of Laemmli (15) with a 3.85% acrylamide stacking gel and a 10% acrylamide separating gel. High-molecular-weight markers were purchased from Bio-Rad.

FHA purification. FHA was purified from *B. pertussis* Tohama by the procedure of Sato et al. (35).

Immunological techniques. Monoclonal antibody P12H3 was kindly provided by C. Parker (9). Polyclonal rabbit antiserum against FHA was prepared by emulsifying FHA in Freund's incomplete adjuvant (ratio, 1:1) in a final volume of 1.0 ml and injecting the emulsion subcutaneously and intramuscularly into 3-month-old chinchilla bastard rabbits (Charles River Wiga GmbH) according to the following regime: day 1, 600 μg; day 30, 300 μg; and day 40, 300 μg. On day 50, the rabbits were sacrificed and the serum was collected, pooled, and stored at −20°C until used. Western blotting (immunoblotting) was carried out essentially as described by Burnette (3). Prestained molecular weight markers were purchased from Bio-Rad.

Immunoelectron microscopy. Cells were fixed with 0.5% formaldehyde and 0.2% glutaraldehyde (final concentrations) in Luria broth for 1 h on ice. After several washings with 10 mM glycine–50 mM potassium phosphate buffer–0.9% NaCl (pH 6.9), cells were embedded by the method of progressive lowering of temperature (27) with Lowicryl K4M resin, modified as follows: (i) the infiltration with 1 part ethanol and 1 part K4M resin was done overnight, (ii) the infiltration with 1 part ethanol and 2 parts K4M resin was done for 12 h, and (iii) the infiltration with pure resin was done for 2 days with several changes of the resin. Recombinant FHA was detected with polyclonal antibodies against FHA that had been cross-absorbed with *E. coli* and *S. typhimurium aroA* and purified by affinity chromatography with protein A–Sepharose CL-4B. Antibodies bound to sample preparations were visualized by incubation with protein A-gold (gold particle size, 10 nm) complexes after preparation of ultrathin sections (postembedding protocol) as previously described (25, 26). The protein A-gold complexes were produced by established procedures (38).

Pulse-chase experiments. Cultures were grown to the exponential phase at 30°C (A_{590}, 0.7). A 0.7-ml sample was removed, and the cells were harvested by centrifugation, resuspended, and washed with 1.0 ml of minimal M9 medium (29). After resuspension in 1.0 ml of M9 medium supplemented with 0.02% each amino acid except for methionine and cysteine, the cells were grown for a further 1 h at 30°C. Cultures were induced at 42°C for 10 min, pulsed with 10 μCi of ^{35}S-methionine (Amersham) for 5 min at 30°C, chased by centrifugation, and resuspended in supplemented M9 medium containing 0.1% cold methionine. Samples were removed at intervals, centrifuged, washed with ice-cold M9 medium, and prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Radiolabeled molecular weight markers were purchased from Amersham.

RESULTS

Mapping of the *fhaB* gene region encoding the epitope recognized by the anti-FHA monoclonal antibody P12H3. To identify the region encoding the epitope recognized by monoclonal antibody P12H3 (which was the reagent used to detect recombinant FHA throughout this work), we cloned subfragments of the 10-kb E6 fragment from pRMB2 (2) into the corresponding sites of plasmid cloning vectors pEX31A and pEX31B to obtain hybrid proteins consisting of the NH_2-terminal 98 amino acids of the MS2 replicase protein fused in-frame to C-terminal fragments of FHA. Hybrid plasmids were subsequently transformed into *E. coli* 537(pCI857^{TS}) and, after induction at 42°C, whole-cell extracts were subjected to Western blot analysis with both

rabbit polyclonal anti-FHA antibody and monoclonal antibody P12H3. The epitope recognized by the P12H3 hybridoma was found to be present on the 1.0-kb *Bam*HI-*Bgl*II fragment of the *fhaB* open reading frame (ORF) (results not shown).

Construction of FHA expression plasmids. To construct expression systems for producing nonfused FHA, we subcloned the E6 DNA fragment into pUC18*Not*I (11) in both orientations with respect to the *lac* promoter present in the vector to produce hybrids pCG3 and pCG2 (Fig. 1A). The hybrid plasmids were transformed into *E. coli* CAG629, EC538, and 876; very weak expression of FHA was detected only with pCG3 in host strain CAG629, and only then after induction with 1 mM IPTG (Fig. 1C, lane 5).

Expression vector pJLA503 (37) was used to construct a system for the direct expression of FHA. This plasmid contains thermoregulated tandem p_R and p_L promoters and the highly efficient *E. coli atpE* translation initiation region (TIR). The original vector multiple cloning site was modified to construct pJLACG1 (Fig. 2A and B). This new expression vector contained sites for cloning of the *fhaB* gene and in-frame stop codons. The start codon of the *fhaB* ORF for our manipulations was selected on the basis of the information published by Domenighini et al. (6).

Hybrid plasmids pCG14 and pCG18 (Fig. 1A) were obtained by subcloning the 5.9-kb *Sph*I-*Bgl*II and 8.4-kb *Sph*I-*Sph*I fragments in-frame with the ATG start codon present in the *Nde*I site and the translational stop codons provided by the linker *Nde*I-*Sal*I (TAA-TAA). Four extra amino acids are encoded between the *Nde*I and *Sph*I sites of the vector and the FHA sequence. In hybrid plasmid pCG27, these four superfluous amino acids were removed by the addition of the *Nde*I-*Sph*I linker (Fig. 2D) to pCG14 upstream of the *Sph*I-*Bgl*II fragment; this linker encodes the first 15 amino acids of FHA and was designed to optimize mRNA structure, as predicted by the Zucker-Stiegler program (50), 50 nucleotides upstream and downstream of position +1 (first nucleotide of the start codon), and to reduce RNA secondary structure stability between the NH_2-terminal coding region and the *atpE* TIR (Fig. 2C and D). Amino acid sequence fidelity was maintained by the mutagenesis strategy, and *E. coli* major amino acid codons were preferentially used. Hybrid plasmid pCG26 was constructed by subcloning the 3.7-kb *Bgl*II-*Eco*RI fragment into pCG27. Hybrid pCG32 was obtained by digestion of pCG27 with *Sph*I and *Eco*RI, removal of the 5.9-kb *Sph*I-*Eco*RI fragment, and introduction of the *Sph*I-*Eco*RI linker (Fig. 2E). This recombinant plasmid (pJLACG1 plus *Nde*I-*Sph*I linker plus *Sph*I-*Eco*RI linker) was digested with *Sph*I, and the 8.4-kb *Sph*I-*Sph*I fragment was introduced.

To determine whether COOH terminus stability affects FHA expression and to attempt to obtain the secretion of FHA, we constructed two further clones in which the C-terminal 23-kDa export signal of the hemolysin A gene (*hlyA*) was inserted in-frame as an *Eco*RI-*Hpa*I insert downstream of the FHA determinant of pCG26 and pCG27 to form pCG28 and pCG29, respectively.

A third expression system used was based on plasmid pT7-7, which contains the T7 RNA polymerase promoter ϕ10 and the translational start site for the T7 10 protein. Hybrids pCG30 and pCG31 were obtained by subcloning of the *Nde*I fragments of pCG29 and pCG28 (the *Nde*I sites are present in linker *Nde*I-*Sph*I and in the sequence of the *hlyA* 23-kDa export signal) into the vector cut with *Nde*I in such an orientation that the ATG site present in the *Nde*I recognition sequence became the start codon. In both cases, the Shine-Dalgarno sequence and ATG start codon were located in regions of strong secondary structure interactions (stability, −7.0 kcal [ca. −29 kJ]/mol) (data not shown).

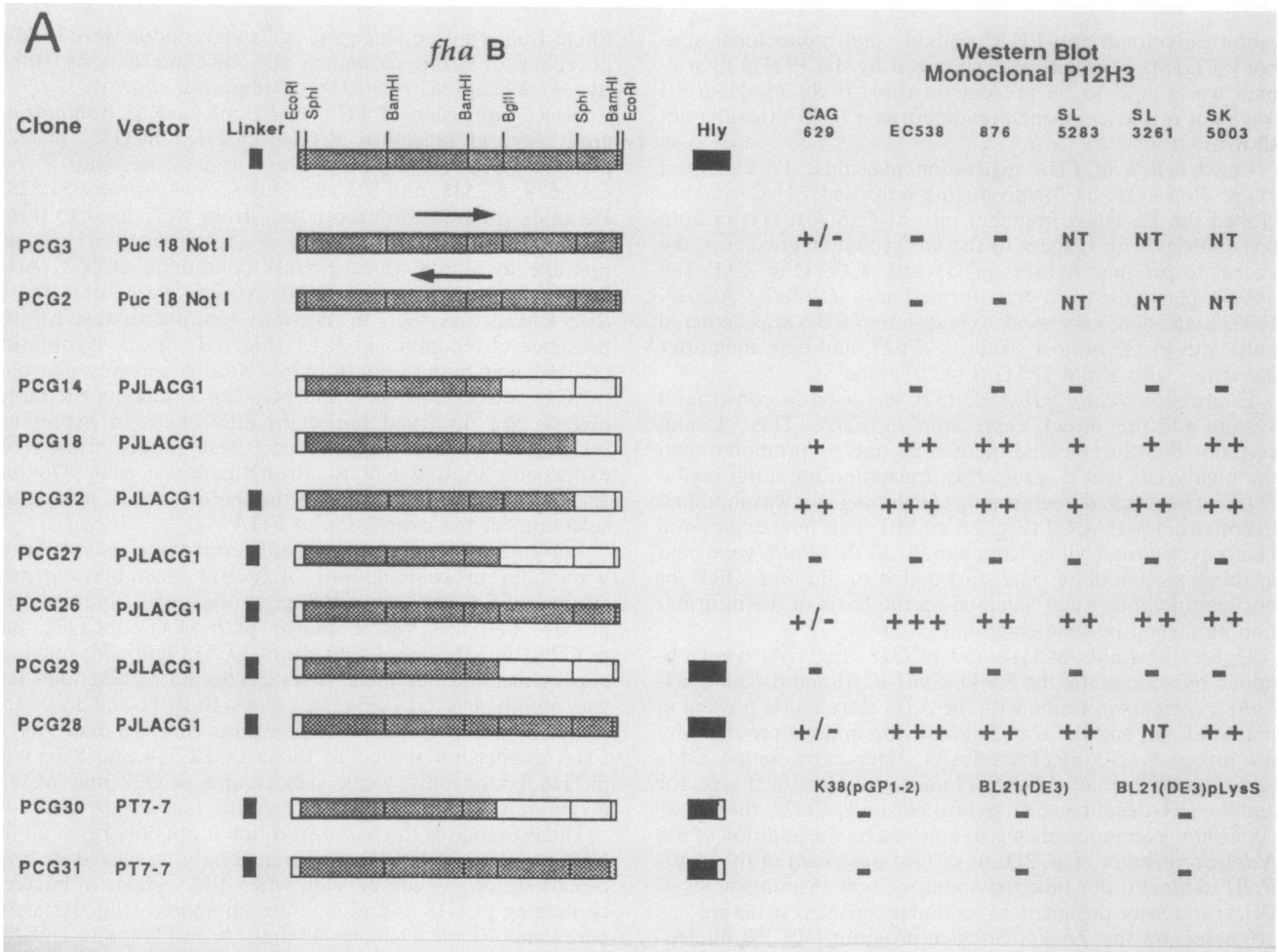
Direct expression of FHA in *E. coli* and *S. typhimurium aroA*. Hybrid plasmids pCG14, pCG18, pCG32, pCG27, pCG26, pCG29, and pCG28 were transformed into *E. coli* CAG629, EC538, and 876 and into *S. typhimurium* SL5283. Plasmids isolated from the latter strain were used to transform *S. typhimurium* SL3261 *aroA*, although it was not possible to obtain transformants containing pCG28. After induction at 42°C, total cellular extracts were subjected to SDS-PAGE and then to Western blotting to test for the presence of recombinant FHA (Fig. 1B and C). Expression of FHA was found only in hybrid strains carrying plasmids (pCG18, pCG32, pCG26, and pCG28) containing the larger inserts. We observed important differences in expression relating to the host strain used; these ranged from weak expression in CAG629 to strong expression in 876 and EC538. These data emphasize the importance of host strain selection for the expression of FHA.

Variations in the expression efficiency were also observed with different combinations of hybrid plasmids and host strains: in CAG629, maximal expression was obtained with pCG18, and less was obtained with pCG3, pCG32, and pCG26 (Fig. 1C, lanes 5 to 8); in EC538 and 876, maximal expression was obtained with pCG26 and pCG32, and less was obtained pCG18 (Fig. 1C, lanes 10 to 12 and 13 to 15). Recombinant *S. typhimurium* strains showed patterns of FHA expression similar to those of EC538 and 876, with pCG18 giving only weak signals and pCG32 and pCG26 giving much stronger signals (Fig. 1C, lanes 16 to 18).

The presence of the *Nde*I-*Sph*I linker not only restored the NH_2 terminus of FHA but also generally increased its level of expression, as can be seen when FHA yields in bacteria containing pCG18 and pCG32 are compared (Fig. 1C, compare lanes 10 and 11, lanes 13 and 14, and lanes 16 and 17). The importance of modifications in the area of the TIR that improve thermodynamic conditions for translation initiation was clearly demonstrated by the results; in pCG18, in which the theoretical value for RNA stability was high (−13.2 kcal [ca. −55.2 kJ]/mol), although both the Shine-Dalgarno sequence and start codon were free of interactions (data not shown), low expression levels were obtained, whereas in pCG32, in which the theoretical value for RNA stability was low (−1.5 kcal [ca. −6.3 kJ]/mol), high expression levels were detected. Alternatively, recombinant protein stability may be improved by the addition of NH_2- and COOH-terminal sequences.

FHA was not detected in the supernatant fluids of cultures of induced *E. coli* cells containing pCG28 and pLG575 (which constitutively express the *hlyB* gene product, necessary for the secretion of fusion proteins containing the *hlyA* C-terminal 23-kDa export signal), nor was the *hlyA* fragment detected in Western blots (data not shown). Therefore, FHA was not secreted by this construct.

Hybrid plasmids pCG30 and pCG31 (pT7-7 expression system) were introduced by transformation into *E. coli* K38λ(pGP1-2), BL21 DE3 (a B strain deficient in *lon* protease and *ompT* outer membrane protease), and BL21 DE3(pLysS) (a strain expressing low levels of T7 lysozyme that inhibit the basal amount of T7 polymerase, which can result in the expression of potentially toxic products in uninduced cells) (41). Induction at 42°C for the first strain and with 1 mM IPTG for the other strains produced no detectable FHA expression (Fig. 1A). The lack of expression may have resulted from strong RNA secondary structure



interactions in the TIR (−7.0 kcal/mol) involving both the Shine-Dalgarno sequence and the start codon and possibly inhibiting translation initiation.

Immunoelectron microscopy demonstrated that in *E. coli* CAG629, 876, and EC538 containing pCG3, pCG18, pCG26, pCG28, and pCG32, recombinant FHA was accumulated in the cytoplasm as a diffuse pattern and inclusion bodies were not observed. In contrast to the results with *E. coli* hybrids, recombinant FHA was deposited in inclusion bodies in *S. typhimurium* SL3261(pCG26) (results not shown).

Investigation of expressing and nonexpressing clones. To investigate the reasons for the differential expression of FHA in different constructs based on the pJLACG1 expression system, particularly factors such as transcription rates, mRNA degradation, and protein stability, we transformed hybrid plasmids pCG14, pCG18, pCG32, pCG27, pCG26, pCG29, and pCG28 into *E. coli* SK5003, which is deficient in polynucleotide phosphorylase and thermolabile for RNase II, the two most important enzymes involved in 3′-to-5′ degradation of mRNA. After simultaneous induction and inactivation of RNase II by shifting of bacterial cultures to 42°C for either 30 min or 1 h, the expression of FHA was observed only from plasmids that had previously given positive results with other host strains (e.g., Fig. 1C, lanes 19 and 20). This result suggests that the instability of mRNA due to 3′-to-5′ exonucleases is not the cause of the poor expression of FHA in some clones.

To determine whether major differences in transcription initiation from the lambda p_R and p_L promoters could explain the differences in FHA expression, we assayed total RNA from CAG629 containing clones pCG27 (negative expression) and pCG26 (positive expression) by primer extension with the FHA*a* probe. In both clones, transcription was directed efficiently by the p_L promoter (Fig. 3). The start of transcription was 173 nucleotides upstream of start codon ATG, in accordance with the p_L start site (37). There was no evidence of another start site further upstream and corresponding to the position of the lambda p_R promoter present in the vector, consistent with the finding that less than 5% of transcripts originated from the p_R promotor in pJLA503 (17a). Thus, poor transcription initiation is not the cause of poor expression of FHA encoded by pCG27.

To determine whether the mRNA was degraded from the 3′ end and whether transcription extended beyond the area complementary to the FHA*a* probe, we bound total RNA from CAG629 containing hybrid plasmids pCG27 and pCG29 (negative expression) and pCG26 and pCG28 (positive expression) to nylon membranes and hybridized it with probes complementary to different areas of the *fhaB* ORF (FHA*a*, FHA*b*, FHA*c*, FHA*d*, FHA*e*, FHA*f*, FHA*g*, and FHA*h*; see Materials and Methods). Hybridization was also carried out with probes complementary to the vector DNA sequence upstream from the start of transcription (jla*501*, negative control) and to the hemolysin 23-kDa COOH-terminal signal (Hly*1*). No hybridization signals were detected with the jla*501*, Hly*1*, or FHA*h* probes with RNA from any of the four strains tested. In contrast, good hybridization signals were obtained with probes FHA*a*, FHA*b*, FHA*c*, FHA*d*, FHA*e*, FHA*f*, and FHA*g* with RNA from all strains (expressing and nonexpressing clones) (Fig. 3C). This result indicates that the major portion of the FHA gene is transcribed and that the resulting mRNA is reasonably stable in all clones.

To analyze possible differences in efficiencies of translation and stability of the translation product, we performed pulse-chase experiments with *E. coli* EC538 containing pCG27 and pCG26. After induction, a 5-min pulse with ^{35}S-methionine, and chases varying from 5 to 60 min with cold methionine, the major 200- to 220-kDa band was clearly seen in EC538(pCG26) and persisted without change for at least 1 h (Fig. 3D, lanes 1 to 4). In corresponding experiments, this band was not detected in cells containing the vector without the insert (data not shown). Analysis of other FHA bands was not possible because of the presence of background proteins encoded by other mRNA species and the appearance and disappearance of other proteins because of the effects of heat shock. The 200- to 220-kDa band was also produced by EC538(pCG27) but was very weak (Fig. 3D, lanes 5 to 8), suggesting differences between pCG26 and pCG27 in mRNA translation efficiencies.

DISCUSSION

FHA is an important adhesin involved in the early interactions between *B. pertussis* and host cells (44, 45). It is a major candidate antigen for incorporation into a new generation of safer vaccines against whooping cough. Until now, however, its production from *B. pertussis* has been problematic with regard to slow growth rates, low yields, and contamination with pertussis determinants involved in adverse reactions. The goal of the present effort has been to determine conditions that allow high-level expression of nonfused FHA in *E. coli*.

The estimated coding region for the 200- to 220-kDa FHA protein secreted from *B. pertussis* is 6.2 kb, approximately the size of the *Sph*I-*Bgl*II fragment. However, the FHA ORF extends over 11 kb. In experiments with *E. coli* and *S. typhimurium* containing pJLACG1-derived hybrid plasmids, only those inserts extending beyond the coding region for the secreted 200- to 220-kDa protein yielded good expression of

FIG. 1. Expression of nonfused FHA in *E. coli* and *S. typhimurium aroA*. (A) Diagrammatic representation of plasmids constructed to investigate FHA expression. The presence or absence of the *Nde*I-*Sph*I linker (see Fig. 2D) is indicated by short solid boxes. Hatched bars correspond to the *fhaB* gene sequences present in different hybrids. The *Eco*RI-*Hpa*I fragment encoding the 23-kDa C terminus of the *E. coli* hemolysin (Hly) is represented by long solid boxes. The level of FHA expression, as determined by Western blot analysis with monoclonal antibody P12H3, ranged from no expression (−) to strong expression (+++). The *Eco*RI-*Nde*I DNA fragment encoding part of the *hlyA* 23-kDa C terminus is shown as partly solid, partly open boxes. Open boxes correspond to the *fhaB* gene sequences not present in different hybrids. Arrows indicate the gene orientation with respect to the *lac* promoter present in vector pUC18*Not*I. NT, not tested. The main restriction sites for genetic manipulations are shown. DNA fragments are not drawn to scale. (B) SDS-PAGE with Coomassie blue staining of total cellular extracts. (C) Western blot analysis with monoclonal antibody P12H3 of total cellular extracts. Lanes: 1, molecular weight standards (in thousands); 2, purified FHA from *B. pertussis* Tohama; 3, *B. pertussis* Tohama; 4 to 8, *E. coli* CAG629 containing pUC18*Not*I (4), pCG3 (5), pCG18 (6), pCG32 (7), and pCG26 (8); 9 to 12, *E. coli* EC538 containing pJLACG1 (9), pCG18 (10), pCG32 (11), and pCG26 (12); 13 to 15, *E. coli* 876 containing pCG18 (13), pCG32 (14), and pCG26 (15); 16 to 18, *S. typhimurium* SL3261 *aroA* containing pCG18 (16), pCG32 (17), and pCG26 (18); 19 and 20, *E. coli* SK5003 containing pCG26 (19) and pCG27 (20). The main Coomassie blue-stained FHA bands are indicated by an arrow. In all cases, inductions were performed at 42°C for 1 h.

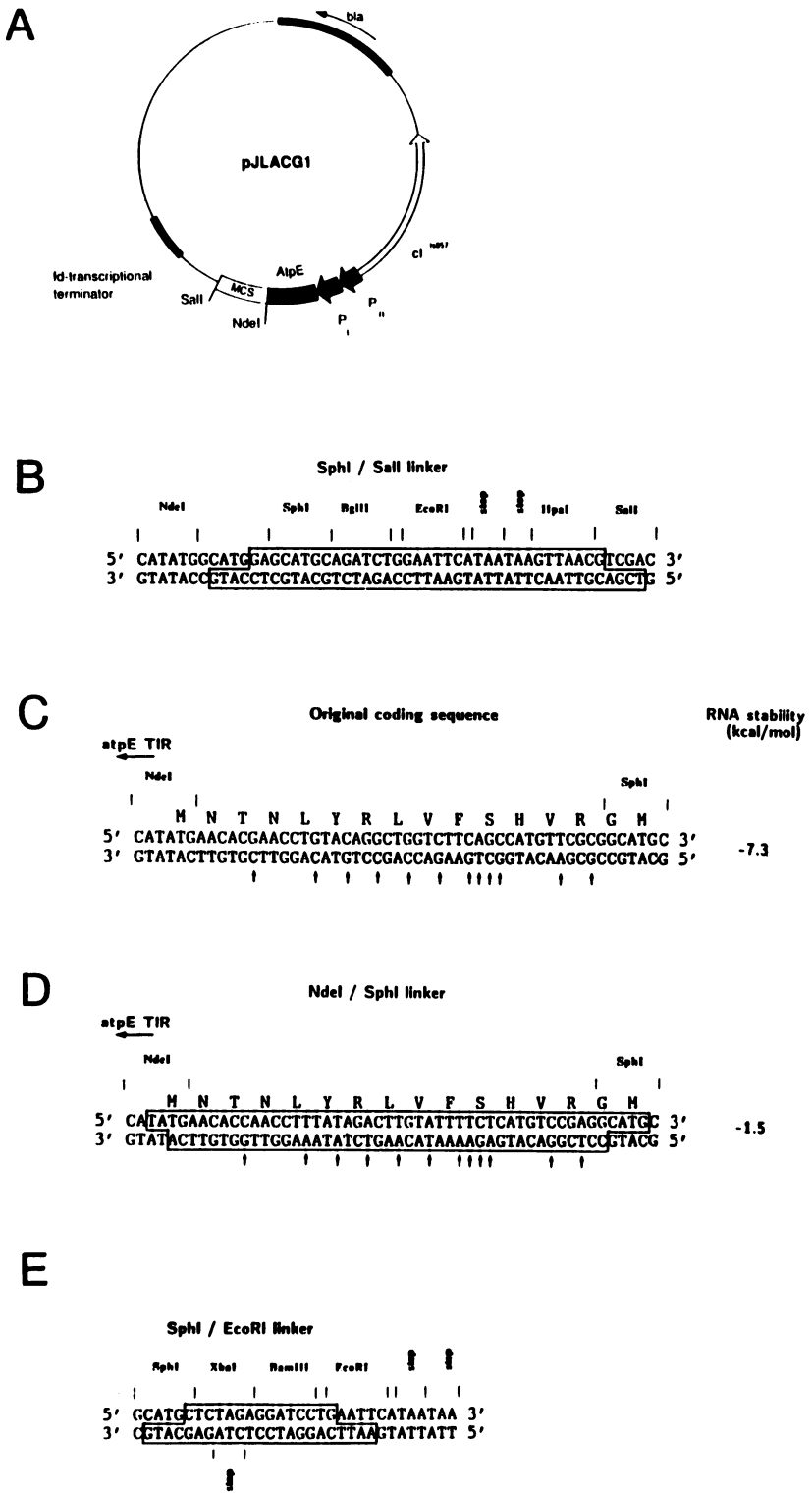


FIG. 2. Construction of plasmids for the direct expression of FHA. (A) Expression plasmid pJLACG1, which contains the lambda p_R and p_L promoters in tandem (solid arrows), the *atpE* TIR (AtpE; solid box), the fd transcriptional terminator, the β-lactamase gene (bla), the thermosensitive repressor (cI^{ts857}), and the *Nde*I-*Sal*I multiple cloning site (MCS; open box). (B) *Sph*I-*Sal*I linker used to modify the MCS of pJLA503 to construct pJLACG1 and allow the subcloning of different DNA fragments encoding FHA into the expression vector. (C and D) Original (C) and modified (D) coding regions of the *Nde*I-*Sph*I linker. The nucleotide sequence of the N-terminal region of FHA is given in one-letter codes. The *Nde*I (CATATG) and *Sph*I (GCATGC) restriction sites are indicated. Base pair changes corresponding to differences between the original nucleotide sequence and the synthetic oligonucleotide linker *Nde*I-*Sph*I are indicated by small arrows; large arrows delineate part of the *atpE* translation initiation region present in expression plasmid pJLACG1. The RNA stability ±50 bases from the ATG start codon was calculated by the method of Zucker and Stiegler (50); 1 cal = 4.184 J. (E) *Sph*I-*Eco*RI linker used to obtain pCG32. Boxes indicate the oligonucleotide linkers.

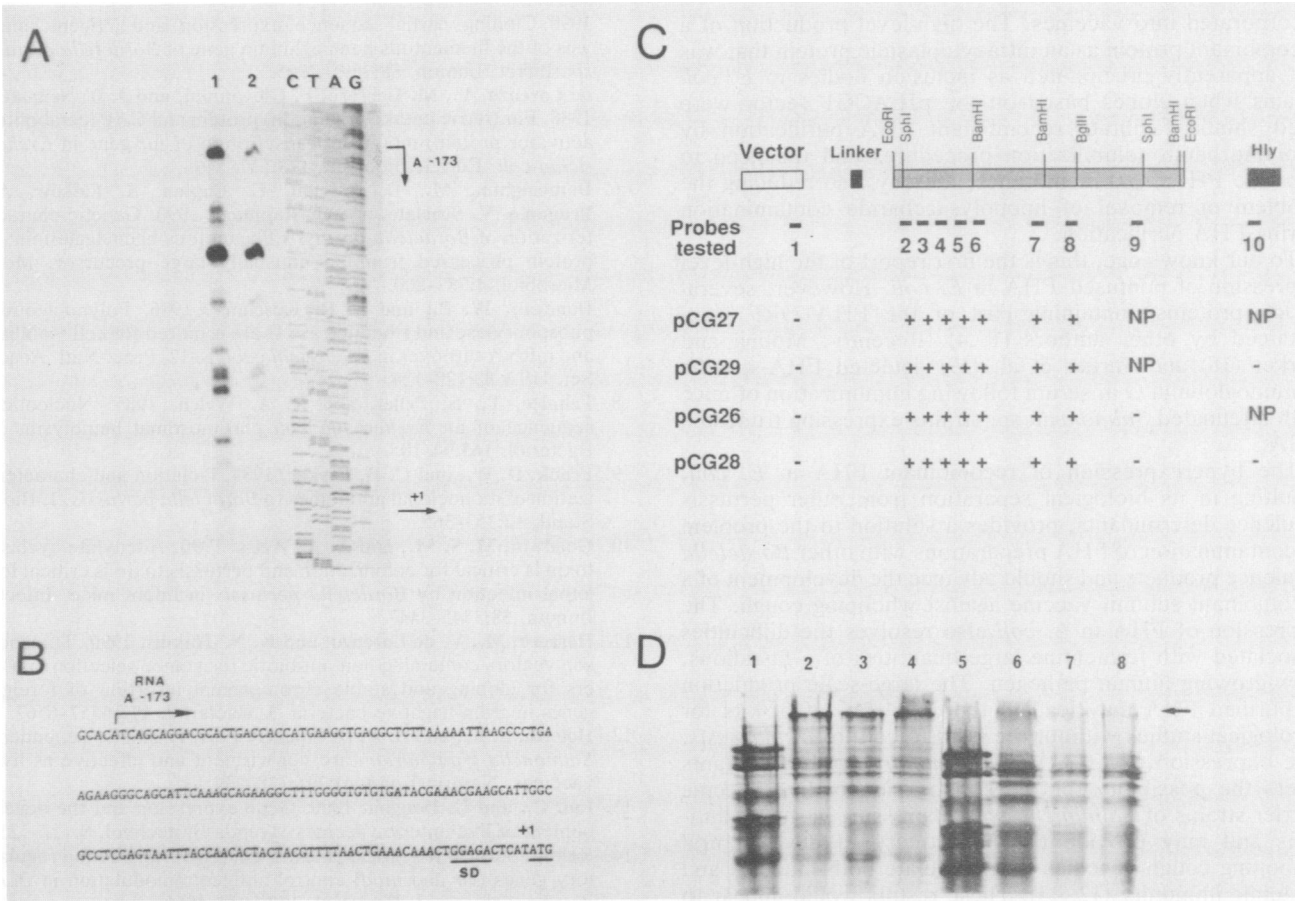


FIG. 3. (A) Reverse transcriptase mapping of the transcriptional start site of the recombinant FHA gene carried by pCG27 and pCG26. Lanes G, A, T, and C contain DNA sequence reactions of pCG27 with the FHA*a* primer. Primer extension with RNA preparations isolated from CAG629 containing pCG27 (lane 1) and pCG26 (lane 2) following induction at 42°C for 30 min was carried out with the FHA*a* primer. The transcriptional start site at base A −173 and the position of the first base in the RNA message and the adenosine (base +1) of the ATG start codon are indicated by arrows. (B) Sequence of the upstream region of the recombinant FHA gene. The transcriptional start site relative to the ATG start codon is indicated by an arrow. The ATG start codon and concensus Shine-Dalgarno region (SD) are underlined. (C) Diagrammatic representation of the results of total RNA dot blot hybridizations of CAG629 containing pCG27, pCG29, pCG26, and pCG28. The probes were jla*501* (1), FHA*a* (2), FHA*b* (3), FHA*c* (4), FHA*d* (5), FHA*e* (6), FHA*f* (7), FHA*g* (8), FHA*h* (9), and Hly1 (10). The probes are shown in approximate correspondence to their positions in the DNA sequence. The *fhaB* gene sequence is indicated as a hatched box. NP, fragment not present in the hybrid. + and − indicate the presence or absence of hybridization. (D) Pulse-chase experiments with *E. coli* EC538 containing pCG26 (lanes 1 to 4) and pCG27 (lanes 5 to 8), corresponding to time zero (uninduced) (lanes 1 and 5) and 10 min (lanes 2 and 6), 20 min (lanes 3 and 7), and 30 min (lanes 4 and 8) after induction. The arrow indicates the main protein product.

FHA. Several experiments were carried out to study the lack of expression of FHA from constructs not containing the COOH-terminal portion of the FHA ORF. The results obtained indicated that mRNA synthesis and stability were not the cause of the difference and that perhaps mRNA translation was lower in constructs with low expression. Interestingly, probes FHA*h* and Hly*1*, specific for the nonexpressed C-terminal portion of the FHA ORF and for the coding region of the C terminus of hemolysin in FHA-hemolysin fusions, respectively, failed to hybridize either to expressing or to nonexpressing clones (Fig. 3C). This result reflected the failure to detect HlyA protein with a specific polyclonal antiserum against the COOH terminus of hemolysin (data not shown) and suggested that in *E. coli* the region downstream of the FHA*g* probe either was not transcribed or was highly unstable. The requirement of this region for FHA expression in *E. coli* is therefore surprising. The DNA fragment present in expressing clones between the FHA*g* and FHA*h* probes may encode a peptide sequence which is involved in the stability of the major FHA band. In cellular extracts of *B. pertussis*, proteins that reacted with antibodies raised against fusion proteins with the COOH-terminal part of the FHA ORF were detected (6). Extracts of *B. bronchiseptica* and *B. parapertussis* did not react with such antibodies. These results highlight the need for further investigations to elucidate the function of the COOH-terminal part of FHA in both *E. coli* and *Bordetella* spp. It would in any case seem that a number of factors, including mRNA instability, the levels of proteases produced in different *E. coli* strains, the preferential degradation of recombinant FHA due to the absence of protein signals found in expressing clones, and the inherent instability of the FHA protein, may strongly influence levels of expression of recombinant FHA.

The expression of hybrid plasmids in strains EC538 and 876 yielded higher levels of FHA than those observed in wild-type *B. pertussis* Tohama. The protein was highly degraded in both strain Tohama and recombinant clones. However, this degradation may not be critical for FHA

incorporated into vaccines. The high-level production of a recombinant protein as an intracytoplasmic protein that was not apparently precipitated as inclusion bodies in *E. coli* strains when clones based on the pJLACG1 vector were used should facilitate recombinant FHA purification by avoiding harsh solubilization procedures and the need to separate FHA from membranes, thereby also reducing the problem of removal of lipopolysaccharide contamination during FHA purification.

To our knowledge, this is the first report of the high-level expression of nonfused FHA in *E. coli*. However, several fusion proteins containing part of the FHA ORF were obtained by other authors (1, 4). Recently, Molina and Parker (18) and Parker et al. (19) detected FHA-specific immunoglobulin G in serum following immunization of mice with attenuated *Salmonella* sp. strains expressing truncated FHA.

The hyperexpression of recombinant FHA in *E. coli*, resulting in its biological separation from other pertussis virulence determinants, provides a solution to the problem of contamination of FHA preparations with other *Bordetella* virulence products and should advance the development of a recombinant subunit vaccine against whooping cough. The expression of FHA in *E. coli* also resolves the difficulties associated with fermenting large quantities of a fastidious, slow-growing human pathogen. The large-scale production of purified FHA may lead to the development of kits for serological studies without the need to culture *B. pertussis*. The expression of FHA in *S. typhimurium aroA* mutants offers the possibility for the construction of live vaccine carrier strains of *Salmonella* spp. expressing pertussis antigens and may provide the opportunity to develop oral whooping cough vaccines that stimulate both mucosal and systemic immunity (12, 13). These results would appear to open new perspectives for the development of both subunit and live vaccines against whooping cough.

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