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Characterization of Three Proteins Expressed from the Virulence Region of Plasmid pSDL2 in *Salmonella dublin*

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Infection of both cattle and humans with *Salmonella dublin* can result in septicemia and death. Like many nontyphoid *Salmonella* species that cause disease, *S. dublin* contains a cryptic plasmid (pSDL2) that is required for the full expression of virulence. Transposon mutagenesis of pSDL2 defined a 4.1-kb EcoRI region that is necessary for the development of a systemic infection in BALB/c mice. This *Eco*RI fragment was cloned into an expression vector (pEL11), and three proteins produced from this region with apparent molecular weights of 30,500, 76,000, and 27,000 were identified. Because bacterial proteins that play a role in virulence are often associated with the outer membrane, we were interested in establishing whether the proteins expressed from the *Eco*RI fragment are located in the membrane. Transposon mutagenesis of pEL11 with Tn*phoA* defined the order of the genes along the fragment and suggested that the proteins may be exported out of the cytoplasm. Sucrose gradient cell fractionation was done to identify the cellular location of each of the three proteins. The 30-kDa protein was identified in the outer membrane fraction, and the 76-kDa protein was located in the cytosolic fraction. The 27-kDa protein was identified in both the cytosolic and the outer membrane fractions. The outer membrane contained less than 10% of the activity of enzymes known to be located in the cytoplasm, periplasm, and inner membrane. Sequence data of the 4.1-kb *Eco*RI region revealed that both the 30- and the 27-kDa proteins lack a typical signal sequence for export out of the cytoplasm (M. Krause, C. Roudier, J. Fierer, J. Harwood, and D. G. Guiney, Mol. Microbiol. 5:307, 1991). The outer membrane location of these proteins suggests that they may be exported out of the cytoplasm by an unusual mechanism.

Many enteric pathogens, including toxigenic and enteroinvasive *Escherichia coli*, *Shigella* species, *Yersinia* species, and nontyphoid *Salmonella* species, contain plasmids that are required to mediate the full expression of virulence. The plasmids of enteroinvasive *E. coli* and *Shigella* species contain genes that promote invasion into the mucosal cells of the gut (15, 36, 39), resulting in an enteritis confined to the bowel. In contrast, chromosomal genes of *Yersinia* and *Salmonella* species promote invasion through the bowel mucosa (6, 29, 32), and their plasmids are responsible for the establishment of a progressive systemic infection in the reticuloendothelial system (4, 5, 10, 12, 16, 41). Little is known about the location, the regulation of expression, or the function of the gene products expressed from *Salmonella* virulence plasmids.

There is convincing evidence that nontyphoid *Salmonella* species harbor a family of plasmids that mediate virulence. In addition to *Salmonella dublin* (5, 16, 44), plasmid-mediated virulence has been reported for *S. typhimurium* (10, 13, 30, 42), *S. enteritidis* (20), *S. cholerae-suis* (17, 21), and *S. gallinarum* (1). Several groups have used deletion analysis and transposon mutagenesis to define the virulence regions within the plasmids of different *Salmonella* serotypes. Restriction mapping and hybridization studies of these virulence regions demonstrate a high degree of homology (2, 35). Roudier et al. evaluated 22 *Salmonella* serotypes for homology to the 4.1-kb *Eco*RI virulence region of pSDL2 by Southern blot hybridization. The nine isolates that hybridized to the *Eco*RI probe were the same isolates that demonstrated a virulent phenotype in BALB/c mice. Similarly, Williamson et al. used an 8-kb fragment from an analogous virulence region of the plasmid harbored in *S. typhimurium* to screen 50 *Salmonella* strains. Forty of the plasmids showed hybridization with the 8-kb fragment (44). More recently, sequence analysis of the 4.1-kb *Eco*RI region of pSDL2 revealed that these genes are nearly identical to the genes of the equivalent region of the plasmid contained in at least one strain of *S. typhimurium* (23).

The studies presented in this work demonstrated that one and possibly two of the proteins expressed from the 4.1-kb *Eco*RI region are located in the membrane. Most bacterial proteins that are exported out of the cytoplasm contain signal sequences that are cleaved during the transmembrane transport across the cytoplasmic membrane. Analysis of the derived protein sequences encoded from the 4.1-kb *Eco*RI fragment failed to predict the presence of signal sequences. The lack of signal sequences would usually suggest that the proteins are located in the cytoplasm. This raises the interesting possibility that these proteins are exported by a unique or unusual mechanism.

MATERIALS AND METHODS

Bacterial strains, plasmid constructs, and phage. The bacterial strains, plasmids, and phage used in these experiments are listed in Table 1. Cells were grown at 37°C in Luria broth (LB) or on LB containing 1.5% (wt/vol) agar. Antibiotics were added for selection at the following concentrations: penicillin, 200 μg/ml; kanamycin, 50 μg/ml; and nalidixic acid, 20 μg/ml.

Construction of the expression vector containing the *Eco*RI fragment and induction of the proteins expressed from the *Eco*RI fragment. The 4.1-kb *Eco*RI fragment was gel purified and cloned into the *Eco*RI site of the expression vector pMMB66EH (7). The orientation of the insertion was con-

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TABLE 1. Bacteria, plasmids, and phage

Strain, plasmid, or phage	Description	Reference or source
Strains		
S. dublin LANE	Wild-type strain, clinical isolate containing pSDL2	16
S. dublin LD842	Derivative of LANE cured of pSDL2	16
S. typhimurium LB5000	Strain LT2 *flaA66 metA22 trp-2 rspL xyl-401 ile-452 leu*, $r^- m^+$, *H1*-b, *H2*-enx	Gift from V. L. Miller
E. coli JA221	*leuB* Δ*trpE5 lacY recA hsdR hsdM*$^+$	8
E. coli C2110	Nal^r *polA his rha*	38
E. coli MV12	C600, Δ*trpE5 recA*; used to maintain helper plasmid pRK2073	18
E. coli HB101	*hsdS recA*, Sm^r	3
Plasmids		
pSDL2	80-kb wild-type virulence plasmid of *S. dublin* LANE	2
pMMB66EH	9.7-kb expression vector containing a polylinker sequence and genes for the *tac* promoter, *lacI*^{q} repressor, and β-lactamase	7
pEL11	13.8-kb construct containing the 4-kb *Eco*RI virulence region cloned into pMMB66EH	This study
pRK2073	Tp^r Sm^r *tra*$^+$, rep ColE1; contains all regions necessary for conjugation	45
Phage λb221 *rex*::Tn*phoA* *c*I857 *P*80	Tn*phoA* is a derivative of Tn*5* containing the Km^r determinant and the *phoA* gene lacking its signal sequence	26

firmed by restriction endonuclease analysis as specified by the vendor (BRL, Gaithersburg, Md.). The resulting plasmid was designated pEL11.

To induce synthesis of the proteins encoded on the *Eco*RI fragment, *E. coli* JA221 carrying pEL11 was grown to the midlog phase with aeration in LB and penicillin at 37°C, and then the *tac* promoter was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 to 4 h.

Expression of new proteins was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole bacterial lysates grown with or without IPTG. The cultures were adjusted to approximately equal optical densities, and the samples were loaded onto the gels under reducing conditions after boiling for 5 min in the presence of 2-mercaptoethanol.

Isolation of DNA. Rapid isolation of plasmid DNA was done according to the directions contained in the GeneClean Kit (Bio 101, La Jolla, Calif.) or by the alkaline lysis method (25) followed by the GeneClean procedure.

Transposon mutagenesis of pEL11 with Tn*phoA*. The *E. coli* HB101(pEL11::Tn*phoA*) constructs were generated by infection of *E. coli* HB101(pEL11) with λ*b*221 *rex*::Tn*phoA* *c*I857 *P*am80 (kindly provided by Virginia Miller, Department of Microbiology and Molecular Genetics, University of California, Los Angeles). Tn*phoA* is a derivative of Tn*5* that contains a gene encoding kanamycin resistance and a truncated version of the gene for alkaline phosphatase (AP) that lacks its promoter, ribosomal binding site, and signal sequence. AP activity is expressed only if the truncated gene fuses downstream from an exogenous signal sequence, which will allow the export of AP out of the reducing environment of the cytoplasm (26, 27). The λTn*phoA*-infected cells were collected by centrifugation, resuspended in 100 μl of LB, and plated on LB agar containing kanamycin to select for Tn*phoA* inserts and containing penicillin to select for pEL11. After 18 h of incubation at 37°C, the plates were scraped and the bacteria were pooled together. To select for pEL11::Tn*phoA*, plasmid DNA was isolated and transformed into *E. coli* HB101 (14). The transformants were plated onto LB agar containing penicillin, kanamycin, 40 μg of 5-bromo-4-chloro-3-indolylphosphate (XP) disodium salt (Sigma, St. Louis, Mo.) per ml, and 100 μM IPTG (Sigma). XP is a chromogenic substrate that turns blue after cleavage by AP. All of the colonies represent successful transposition events of Tn*phoA* into pEL11. The presence of blue colonies implies that the *phoA* gene has fused to a gene containing a signal sequence. Blue colonies were selected at 48 h.

Alternatively, the pooled infected bacteria were mated by the triparental mating method (5) with *E. coli* C2110 (Nal^r), and the helper plasmid pRK2073 maintained in *E. coli* MV12. Samples of approximately 1 ml of overnight cultures of each of the bacteria noted above were pooled and filtered through a 45-μm-pore-size filter. The filter was placed on a LB agar plate without selection; after 4 h of incubation at 37°C, the bacteria were washed from the filters with 0.9% NaCl and plated onto LB agar with kanamycin, penicillin, nalidixic acid, XP, and IPTG. Blue colonies were selected at 48 h for further characterization.

Cell fractionation. The periplasmic fraction was collected by a modification of the method of Manoil and Beckwith (27), and the remaining fractions were obtained by the method of Schnaitman (37), with some modifications. Late-log-phase cultures of bacteria were inoculated (1:100) into fresh LB and incubated for 1 to 2 h at 37°C and then grown overnight with or without IPTG (1 mM). Cultures were adjusted to an optical density of 1.0, sedimented, and suspended in spheroplast solution (100 mM Tris, 0.5 mM EDTA, 0.5 mM sucrose [pH 8.0]). After centrifugation, the periplasmic fraction was released by exposing the cells to a hypoosmotic shock with double-distilled H_2O and 1 mM $MgCl_2$. The periplasm was collected after centrifugation and stored at 4°C. The cells were washed once in 50 mM Tris (pH 7.4), resuspended in 10 mM Tris (pH 7.8)–0.75 M sucrose, and burst open by passing through a French press (10,000 lb/in^2) three times. Unbroken cells were removed by two slow centrifugations (10 min at 5,000 × *g*). The concentration of the buffer was adjusted to 40 mM Tris (pH 7.4), and an aliquot of the broken cell lysate was saved at 4°C for enzyme assays (described below). The lysate was then sedimented in a Sorvall Ultraspeed OTD75B centrifuge at 4°C for 2 h at 250,000 × *g* (Sorval T-865 rotor). The soluble fraction (cytoplasm) was stored at 4°C, and the membrane fraction was resuspended in 25% sucrose–5 mM EDTA (pH 7.4) and loaded onto a 55 to 30% sucrose gradient. The membranes were subjected to centrifugation through the sucrose gradients at 4°C for 48 to 60 h at 206,000 × *g* (Sorval

T-865 rotor), and the gradients were fractionated with a peristaltic pump. The inner and outer membranes were suspended in 10 mM Tris (pH 8.0)–5 mM EDTA buffer.

Protein assays. The protein concentration of each fraction (cytoplasm, periplasm, inner and outer membranes) and the aliquot containing the broken-cell lysate, was determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.).

Enzyme assays. Each cellular fraction was tested by using standard assays for an enzyme characteristically located in the cytoplasm, periplasm, and the inner membrane. All assays were performed at 24°C in a Beckman DU-70 spectrophotometer. Contamination of fractions with outer membrane was assessed by the presence of one of the *Salmonella* major outer membrane proteins.

(i) LDH (EC 1.1.1.27) (cytoplasmic marker). Lactate dehydrogenase (LDH) activity was determined by measuring the oxidation of NADH in the presence of pyruvate (decrease in A_{340}) (40). The incubation mixtures contained 0.1 M sodium phosphate buffer (pH 7.0), 0.2 mM NADH, 10 mM pyruvate, and the sample (2 to 40 μg of protein) in a volume of 1 ml. The final levels of activity were determined after subtraction of the NADH oxidase activity described below.

(ii) NADH oxidase (inner membrane marker). NADH oxidase activity was determined by the oxidation of NADH by using the same method as that used for LDH activity, except that pyruvate was not added (modified from the procedure in 31).

(iii) β-Lactamase (periplasmic marker). β-Lactamase activity of the sample (2 to 20 μg of protein) was determined by decolorization (decrease in A_{600}) of a starch-iodine solution in the presence of 0.2 mM ampicillin (34).

SDS-PAGE and Western immunoblot analysis. SDS (8 or 10%)–polyacrylamide gels (24) were used for the separation of *Salmonella* (30% acrylamide, 1.6% bisacrylamide), and *E. coli* (28% acrylamide, 0.8% bisacrylamide) proteins. The whole bacterial lysates were adjusted to equivalent optical densities, and then 10 to 15 μl of a 10-fold-concentrated suspension was loaded per lane. Approximately 40 μg of protein per lane was loaded for each of the cellular fractions. All samples were prepared under reducing conditions in the presence of 2-mercaptoethanol and boiled for 3 to 5 min. Gels were stained with Coomassie brilliant blue R (Sigma). For Western blot analysis, the proteins were separated by SDS-PAGE and then transferred electrophoretically (Trans-Blot Cell; Bio-Rad) to nitrocellulose paper (Sartorius, West Coast Scientific, Inc., Emeryville, Calif.). Nonspecific antibody binding was blocked by incubating the nitrocellulose in 3% (wt/vol) dry milk in TSA buffer (63 mM Tris [pH 7.4], 0.15 M NaCl, 0.1% [wt/vol] sodium azide). The blots were probed with polyclonal rabbit serum generated by immunization of a rabbit with partially purified preparations of each of the three proteins expressed from the 4.1-kb *Eco*RI fragment (unpublished data). Binding of the primary antibody was detected by using goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Cappel, West Chester, Pa.) and then incubating the preparation in a reaction mixture for alkaline phosphatase containing 0.5% (wt/vol) XP *p*-toluidine salt (Sigma) and 0.1% (wt/vol) nitroblue tetrazolium (Sigma) in 50 mM Tris–3 mM $MgCl_2$ [pH 10].

For Western blots of the fusion proteins (*E. coli* pEL11::Tn*phoA*), the primary antibody was mouse anti-alkaline phosphatase (*E. coli*) antibody (Caltag Laboratories, San Francisco, Calif.) and the secondary antibody was goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Cappel).

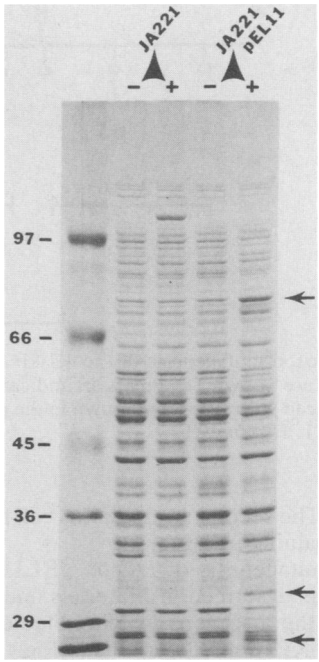

FIG. 1. Induction of the proteins expressed from the 4.1-kb *Eco*RI fragment and subjected to 10% SDS–PAGE. Whole bacterial lysates of *E. coli* JA221 without and with the plasmid pEL11 are indicated; molecular weight markers are shown on the left. After growth to the midlog phase, the bacteria were grown without (−) and with (+) 1 mM IPTG for 3 to 4 h at 37°C. The three proteins indicated by the arrows were present when bacteria containing the plasmid were induced with IPTG and were not present in bacteria without the plasmid.

RESULTS

Previous studies performed in this laboratory with transposon mutagenesis of p9-18Δ7 identified a 4.1-kb *Eco*RI fragment within the *Sal*I-B region that is essential for the full expression of virulence in BALB/c mice (5a, 5b). p9-18Δ7 is a deletion derivative of the native plasmid of *S. dublin* that contains all of the necessary replication and virulence functions (5). To define the protein products encoded on the *Eco*RI fragment, this fragment was cloned onto the expression vector pMMB66EH, which contains the *tac* promoter (7). This construct, designated pEL11, was transformed into *E. coli* JA221. When grown in the presence of IPTG, *E. coli* JA221(pEL11) synthesized three new proteins with apparent molecular weights of approximately 76,000, 30,500, and 27,000 (Fig. 1). These proteins were not produced by *E. coli* JA221 or by uninduced *E. coli* JA221(pEL11). Expression of the proteins occurred when the *Eco*RI fragment was inserted in one orientation only. Approximately 88% of the coding capacity of pEL11 was accounted for by these three proteins. Proteins of similar sizes were predicted by the recently published DNA sequence of this *Eco*RI fragment. The DNA sequence revealed three open reading frames, which predicted the presence of three proteins with molecular weights of 28,100, 65,500, and 27,600 (23).

Because many bacterial proteins involved with virulence are located in the outer membrane, we were interested in determining whether any of the proteins encoded by the *Eco*RI fragment was an outer membrane protein. Two separate strategies were used to evaluate this question. The first was to generate alkaline phosphatase fusions with the trans-

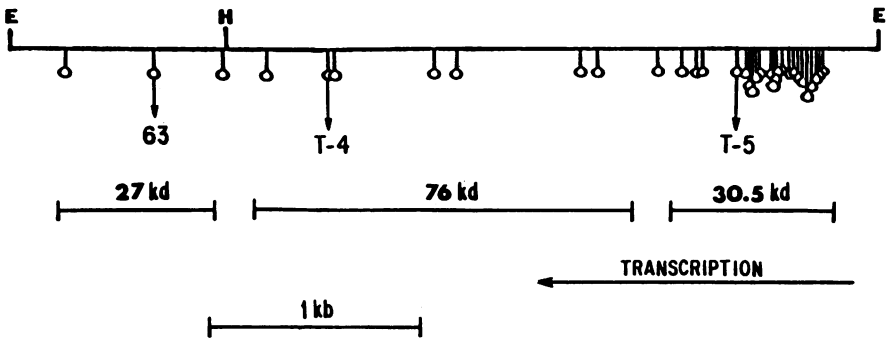

FIG. 2. Transposon mutagenesis of the 4.1-kb *Eco*RI fragment by Tn*phoA*. The direction of transcription and the order of the proteins along the plasmid are shown. The markers indicate the locations of the Tn*phoA* insertions, which resulted in a blue phenotype when the bacteria were induced with IPTG and grown in the presence of XP. The insertions indicated by the vertical arrows are discussed in the legend to Fig. 3. E, *Eco*RI; H, *Hin*dIII.

poson Tn*phoA*. The second strategy was cell fractionation of *Salmonella* containing pEL11.

Transposon mutagenesis of *E. coli* pEL11 with Tn*phoA* was performed as described in Materials and Methods, and the locations of the insertions yielding blue colonies on LB agar containing XP were determined (Fig. 2). Restriction endonuclease mapping of Tn*phoA* insertions in pEL11 revealed that 92% of the 129 colonies evaluated contained Tn*phoA* insertions into the *Eco*RI fragment. Although there was a clustering of insertions in the first kilobase from the right side of the *Eco*RI fragment, inserts of Tn*phoA* were found along the entire length of the *Eco*RI fragment. To confirm the presence of the fusion proteins, whole bacterial lysates of *E. coli*(pEL11::Tn*phoA*) grown with or without IPTG were evaluated by SDS-PAGE (Fig. 3A). As is clearly evident, novel proteins were synthesized by *E. coli* carrying pEL11::Tn*phoA* derivatives. These fusion proteins were of the sizes that would be predicted from their locations along the map, and they were produced only when expression from the *tac* promoter was induced with IPTG. Mouse anti-alkaline phosphatase (*E. coli*) antibodies were used to verify the presence of the fusion proteins (Fig. 3B). Several other pEL11::Tn*phoA* derivatives were tested with similar results. Some of the inserts did not result in stable fusions (Fig. 3A, lanes 7 and 8), although the presence of alkaline phosphatase was documented by using Western blotting (data not shown).

The Tn*phoA* constructs also allowed us to predict that the order of the proteins from right to left along the fragment was 30.5, 76, and 27 kDa (Fig. 2). Evaluation of the protein products resulting from expression of Tn*phoA* insertions within 0.66 kb from the right end of the *Eco*RI fragment revealed only the presence of a fusion protein but none of the original three proteins (Fig. 3, lanes 3 and 4). Evaluation of the proteins expressed from the pEL11::Tn*phoA* (T-4) construct located at 2.6 kb from the right side of the *Eco*RI fragment revealed the presence of the 30.5-kDa protein as well as a fusion protein of approximately 108 kDa (Fig. 3, lanes 5 and 6). Because approximately 47 kDa of the fusion protein was accounted for by alkaline phosphatase, the remaining 61 kDa of the fusion protein indicated that the 76-kDa protein was probably the second protein ordered along the *Eco*RI fragment. As mentioned above, SDS-PAGE evaluation of constructs containing insertions of Tn*phoA* into the gene encoding the third protein did not reveal any fusion proteins, but the 30.5- and 76-kDa proteins reappeared (Fig. 3, lanes 7 and 8). Again, the order of the proteins along the fragment that we predicted agreed with the sequence data discussed previously (23).

The generation of unstable fusion proteins resulting from gene fusion with Tn*phoA* has been reported previously (26). Many of the gene fusions appeared somewhat unstable in that serial passage of the constructs resulted in a less intense blue phenotype. This did not allow accurate quantitation of the amount of AP activity produced after induction of the *tac*

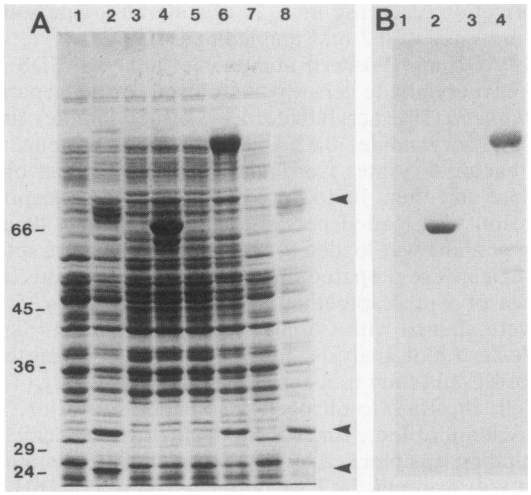

FIG. 3. Induction of alkaline phosphatase fusion proteins. One insertion into each of the genes is shown. Fusion proteins of predicted sizes were produced for constructs T-5 and T-4 in the first and second gene, respectively. Construct 63, located in the third gene, did not result in a stable fusion protein, but the first two proteins encoded from right to left along the *Eco*RI fragment are present (A) *E. coli* pEL11::Tn*phoA* proteins subjected to 10% SDS–PAGE. Samples in odd-numbered lanes in both panels were from cells grown without IPTG; those in even-numbered lanes were from cells grown with IPTG. Lanes 1 and 2, *E. coli* JA221(pEL11); 3 and 4, *E. coli* HB101(pEL11::Tn*phoA*) (T-5); 5 and 6, *E. coli* HB101(pEL11::Tn*phoA*) (T-4); 7 and 8, *E. coli* JA221(pEL11::Tn*phoA*) (construct 63). See Fig. 2 for the location of the inserts along the *Eco*RI fragment. The arrows indicate the proteins expressed from the 4.1-kb *Eco*RI fragment. Western blot analysis confirmed the specificity of the putative fusion proteins. (B) Western blot analysis. Lanes: 1 and 2, *E. coli* HB101(pEL11::Tn*phoA*) (T-5); 3 and 4, *E. coli* HB101(pEL11::Tn*phoA*) (T-4). Anti-alkaline phosphatase antibodies were used to probe the blot.

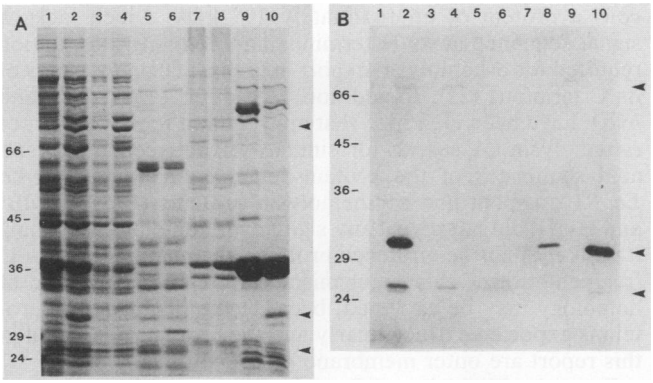

FIG. 4. Cell fractionation of *S. typhimurium* LB5000(pEL11). Sucrose gradient cell fractionation of *S. typhimurium* LB5000 (pEL11) revealed that the 76-kDa protein was located in the cytoplasm and that the 30.5-kDa protein was located in the outer membrane. The 27-kDa protein was identified in both the cytoplasm and the outer membrane (see arrows). (A) Proteins subjected to 8% SDS-PAGE. Samples in odd-numbered lanes were from cells grown without IPTG; those in even-numbered lanes were from cells grown with IPTG. Lanes: 1 and 2, *S. typhimurium* LB5000(pEL11), whole bacterial lysates; 3 and 4, cytoplasmic fractions; 5 and 6, periplasmic fractions; 7 and 8, inner membrane fractions; 9 and 10, outer membrane fractions. Approximately 40 μg of protein per lane was loaded for each of the cell fractions. Western blot analysis was done to verify the specificity of the proteins visualized on the SDS gel. (B) Western blot of Cell Fractionation. The lanes correspond to those described above. The blot was probed with pooled rabbit polyclonal antisera against the three proteins expressed from the 4.1-kb *Eco*RI fragment.

promoter in the original colonies. The initial results were compelling enough to seek additional evidence that the proteins expressed from the 4.1-kb *Eco*RI fragment are membrane-associated proteins.

To further address the question of whether the proteins expressed from the 4.1-kb *Eco*RI fragment were exported out of the cytoplasm, cell fractionation studies were performed. *S. typhimurium* LB5000(pEL11) cells grown with and without IPTG were broken open with a French press, and the membrane fractions were separated by ultracentrifugation through sucrose gradients as described in Materials and Methods (Fig. 4A). The 30-kDa protein and the 27-kDa proteins were seen in the outer membrane fraction, and the 76-kDa protein was seen only in the cytosolic fraction. To establish the specificity of the proteins identified on the stained gels and to increase the sensitivity of the fractionation studies, Western blot analysis of the cell fractions was done with pooled polyclonal rabbit serum containing antibodies against each of the three proteins (Fig. 4B). The results of the Western blots confirmed the location of the 30- and 76-kDa proteins in the outer membrane fraction and the cytoplasm, respectively, and identified the 27-kDa protein in both the cytoplasm and the outer membrane. Similar results were obtained in a second experiment with *S. typhimurium* LB5000(pEL11) and *S. dublin* LD842(pEL11) (data not shown).

The purity of the various cellular fractions was evaluated by measuring the activity of enzymes characteristically found in the cytoplasm (LDH), periplasm (β-lactamase), and inner membrane (NADH oxidase). Approximately 80% of the LDH activity and 30% of the NADH oxidase activity were present in the supernatant (cytoplasmic fraction), compared with the activity measured in the lysate containing the broken cells before ultraspeed centrifugation. The total activity recovered was determined by adding the activities measured in the cytoplasm and in the inner and outer membranes. The cytoplasm contained approximately 65 and 5% of the total LDH and NADH oxidase activities recovered, respectively. The inner membrane contained approximately 31 and 88% of the LDH and NADH oxidase activities, respectively, whereas the outer membrane contained less than 10% of the activities of both the cytoplasmic and inner membrane enzymes. The periplasm contained greater than 88% of the activity for β-lactamase and always contained less than 10% of the activity for the cytoplasmic and inner membrane enzyme markers. Similar results were obtained with four separate cell fractionation procedures. Thus, although there was cross-contamination of the cytoplasm and the inner membrane, the outer membrane was relatively cleanly separated from the other fractions.

The presence of outer membrane proteins in the other fractions was determined by inspection of the gels for one of the major outer membrane proteins of *S. typhimurium* (Fig. 4A, lanes 7 and 8). The inner membrane contained a small amount of contamination with the outer membrane when judged by this criteria.

When the cellular locations of the proteins of interest are interpreted in the context of the results of the enzyme assays, it can clearly be stated that the 30-kDa protein was located in the outer membrane and that the 76-kDa protein was located in the cytoplasm. The 30-kDa protein was found predominantly in the outer membrane fraction, which contained less than 10% contamination from the other fractions. The exact location of the 27-kDa protein, which was identified in both the cytoplasm and the outer membrane, remains to be elucidated.

DISCUSSION

Several groups have demonstrated that the plasmids of *S. dublin* and *S. typhimurium* allow the development of a progressive systemic infection in susceptible hosts. There is convincing evidence that the products encoded on the 4.1-kb *Eco*RI fragment of pSDL2 investigated in these studies are at least in part responsible for the expression of this invasive phenotype. Seventeen separate insertions of Tn*1725* into the *Eco*RI fragment markedly decreased the expression of virulence in BALB/c mice (5a, 5b). In addition, the generation of deletion derivatives from the plasmids contained in several nontyphoid *Salmonella* serotypes demonstrated that the genes responsible for the invasive phenotype are clustered within a small region of the plasmid, which includes the 4.1-kb *Eco*RI fragment. Krause et al. recently reported that all of the genes necessary to express a lethal phenotype in BALB/c mice are located on an 8.2-kb region within the *Sal*I B fragment of the 80-kb native plasmid pSDL2 contained in *S. dublin* (23). Likewise, Gulig and Curtiss were able to express the full virulent phenotype by using an approximately 14-kb fragment of the 100-kb native plasmid of *S. typhimurium* SR-11. Using transposon mutagenesis with Tn*5*, they correlated the presence of a region of the plasmid (pYA403) encoding a 28-kDa protein (VirA) with the ability of the bacteria to infect the spleens of BALB/c mice after oral inoculation (11). Analysis of the DNA sequences revealed that the VirA contained on the virulence plasmid of *S. typhimurium* corresponds to the gene encoding the 27-kDa protein evaluated in this work (9, 23).

Little is known about the cellular location, the regulation of expression, or the function of the proteins expressed from

the virulence regions of the plasmids contained in nontyphoid *Salmonella* species. By cloning the highly conserved 4.1-kb *Eco*RI fragment from the virulence plasmid of *S. dublin* into an expression vector, we identified three proteins expressed from this region. Transposon mutagenesis of the *Eco*RI fragment with Tn*phoA* confirmed the order of the proteins along the DNA fragment (30.5, 76, and 27 kDa), in agreement with the nucleotide sequence data. The Tn*phoA* mutants also suggested that the proteins may be exported out of the cytoplasm by the expression of a blue phenotype (AP^+) when the bacteria were induced by IPTG and grown in the presence of XP on LB plates.

To further clarify whether the proteins are exported out of the cytoplasm and to more clearly determine the cellular location of the proteins, cell fractionation experiments were done with sucrose gradient centrifugation. The cell fractionation studies demonstrated that the 30-kDa protein was located in the outer membrane and that the 76-kDa protein was located in the cytoplasmic fraction when these proteins were overexpressed in *S. typhimurium*. The cellular location of the 27-kDa protein, which appeared in both the cytoplasm and the outer membrane fraction, was not definitively determined by these studies. The specificities of the proteins expressed from the *Eco*RI fragment and identified in the cellular fractions noted above were verified by using rabbit polyclonal antibodies generated against partially purified derivatives of the proteins. The location of the 30-kDa protein could not be accounted for by contamination of the outer membrane with proteins from the other fractions because the outer membrane contained less than 10% of the activity of enzyme markers from the cytoplasm, periplasm, and inner membrane.

The discrepancy between the cytoplasmic location of the 76-kDa protein defined by the cell fractionation studies and the membrane location predicted by the Tn*phoA* insertions into the gene encoding this protein may be due to its hydrophobic N terminus. It is possible that the 76-kDa protein is associated with the inner membrane but does not translocate across it, resulting in the cytoplasmic localization during cell fractionation.

The 76-kDa protein expressed from the 4.1-kb *Eco*RI fragment sometimes appeared as a doublet on the SDS gels, with a larger form appearing at approximately 79 kDa (Fig. 4A, lanes 2 and 4). In addition, the polyclonal rabbit serum recognized two forms of this protein (Fig. 4B, lane 2), as seen on the Western blot analysis. The observation of two forms of this protein on the SDS gel, along with the predicted molecular weight of 65,000 from the nucleotide sequence, suggest that the 76-kDa protein is processed or modified.

Most bacterial proteins that cross the inner membrane utilize the *secA secY* general secretory pathway (reviewed in reference 33). These secreted proteins contain a 15- to 20-amino-acid signal sequence at the N terminus that is cleaved from the protein during the process of transmembrane transport. Interestingly, the nucleotide sequences (23) of the genes encoding the 27- and 30.5-kDa proteins discussed in this manuscript do not predict the presence of classic signal sequences. This raises the question of whether the 30-kDa protein and possibly the 27-kDa proteins may be exported out of the cytoplasm by a unique or unusual mechanism that is independent of the general secretion pathway.

A growing number of proteins have been characterized that lack typical signal sequences and are exported out of the cell (reviewed in references 19 and 43). The prototype for this alternate pathway of secretion is the α-hemolysin of *E. coli*, a protein of approximately 107 kDa, which lacks a signal sequence at its N terminus and contains information required for α-hemolysin export in the last 50 amino acids of its C terminus (22). In addition, two other genes, *hylB* and *hylD*, have been identified that are required for the export of α-hemolysin. A search for similarities between the amino acid sequences of the proteins encoded from the 4.1-kb *Eco*RI fragment and the hemolysin proteins (HylA, HylB, and HylD) did not reveal any significant homologies, making it unlikely that the virulence proteins encoded on the *Eco*RI fragment utilize this mechanism for export. The lack of homology may be expected, because the hemolysin A protein is exported extracellularly and the proteins discussed in this report are outer membrane proteins.

Recently, Michiels and Cornelis (28) reported several Yop proteins (YopH, YopE, YopQ) expressed from the *Yersinia* virulence plasmid that lack signal sequences and that seem to be secreted by a mechanism distinct from both the general secretory pathway and the α-hemolysin pathway. The secretion information is not contained in the C terminus but has been localized to the N terminus of the protein. Curiously, the YopH, YopE, and YopQ proteins do not show regions of strong hydrophobicity at their N termini (28). A search for sequences similar to these Yop proteins and the proteins expressed from the 4.1-kb *Eco*RI fragment also failed to reveal significant homology.

The export and secretion of bacterial proteins are areas of intensive investigation. The existence of at least one mechanism other than the general secretory pathway is clearly established, and evidence for the existence of other pathways is rapidly accumulating. Our observation that the 30-kDa protein and possibly the 27-kDa protein are outer membrane proteins represents another example of a secreted protein(s) without classic signal sequences. Further investigation is needed to determine whether these proteins are secreted by one of the more recently defined pathways or by a unique pathway.

The studies presented in this work are the first to demonstrate the outer membrane localization of one of the virulence proteins expressed from the plasmid in *S. dublin*. The polyclonal antibodies generated against these proteins provide a powerful tool for addressing questions of regulation of expression and function of these highly conserved virulence proteins.

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