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Expression Profile and Subcellular Location of the Plasmid-Encoded Virulence (Spv) Proteins in Wild-Type *Salmonella dublin*

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The plasmid-encoded virulence genes (*spvABCD*) in nontyphoid *Salmonella* strains mediate lethal infections in a variety of animals. Previous studies have shown that these genes are transcriptionally regulated by stationary-phase growth. We studied the expression profile and the subcellular locations of the SpvABCD proteins in wild-type *S. dublin* by using polyclonal antibodies against SpvA, SpvB, SpvC, and SpvD. The cellular levels of the individual proteins were determined during growth by quantitative immunoblotting. As expected, SpvA, SpvB, SpvC, and SpvD were not detectable before the late logarithmic growth phase and appeared in the sequence SpvA, SpvB, SpvC, and SpvD. In contrast to the transcriptional regulation, however, SpvA and SpvB reached their maximal expression shortly after induction and declined during further growth whereas SpvC and SpvD expression remained high throughout the stationary phase, indicating that the Spv proteins are individually regulated at a posttranscriptional level. To localize SpvABCD within the bacteria, the cells were fractionated into the periplasmic, cytoplasmic, inner membrane, and outer membrane components. The cell fractions and the culture supernatant were analyzed by immunoblotting. SpvA was present in the outer membrane, SpvB was present in the cytoplasm and the inner membrane, and SpvC was present in the cytoplasm. SpvD was secreted into the supernatant; however, a substantial portion of this protein was also detected in the cytoplasm and membranes. The molecular weights of SpvD in the supernatant and in the cytoplasm appeared to be equal, suggesting that SpvD is not cleaved upon secretion.

Certain nontyphoid *Salmonella* species harbor large virulence plasmids that mediate systemic and lethal infections in domestic animals including cattle, fowl, and pigs (2, 4, 16, 46). Studies in mice infected by the oral route have shown that these plasmids are not required for invasion of the intestine but promote bacterial survival and proliferation in the reticuloendothelial tissues of the liver and spleen (17, 19, 20). Although the plasmids are not identical among the various *Salmonella* serovars, the 8-kb region required for virulence is highly conserved (15, 18). It contains a cluster of four structural genes, *spvA*, *spvB*, *spvC*, and *spvD*, that are transcribed as an operon and are regulated in vitro by the stationary growth phase and by the intracellular milieu of eukaryotic cells (9, 11, 13, 31). Two bacterial regulatory factors play a pivotal role in the control of *spv* expression. One is RpoS, a σ-subunit of the RNA polymerase that governs the expression of various stationary-phase-induced genes (12, 21, 29). RpoS is responsible for induction of the second regulator, SpvR, which is encoded by the virulence plasmid and belongs to the LysR/MetR-type family of transcriptional activator proteins (2, 6, 9, 25, 37, 41). Both RpoS and SpvR induce *spvABCD* transcription at the *spvA* promoter, resulting in *spvA*, *spvAB*, *spvABC*, and *spvABCD* mRNAs in decreasing order of abundance (7, 14, 22, 23, 27).

Analysis of mRNA and *spv*::*lacZ* reporter gene expression has led to a detailed knowledge about the regulation of the *spv* genes; however, the function of the SpvABCD proteins and the mechanism by which they interact with the host tissue are currently not understood. The derived products SpvA, SpvB, SpvC, and SpvD have predicted sizes of 28, 65, 28, and 25 kDa, respectively, and their identity has been confirmed by overexpression and N-terminal sequencing in a variety of systems (33, 39, 43, 47). While for SpvA, SpvC, and SpvD, no homologies with known proteins were found in the databases, the middle portion of SpvB shows some sequence similarity to the 96-residue Ace peptide of *Vibrio cholerae*, which acts as an ion transporter across cell membranes (42).

The purpose of this work was to gain a better understanding of the function of the structural Spv proteins. We generated polyclonal antibodies against SpvA, SpvB, SpvC, and SpvD and determined their cellular levels in wild-type *S. dublin* during growth by quantitative immunoblotting. We found that SpvA and SpvB levels declined after they had reached their maximum in early-stationary-phase cultures, clearly contrasting with the course of SpvC and SpvD, whose levels increase throughout stationary-phase growth. Analysis of the subcellular localization of SpvABCD within wild-type *S. dublin* revealed that SpvA is exported into the outer membrane, SpvB and SpvC remain in the cytoplasm, and SpvD is secreted into the supernatant. We propose that the individual Spv proteins may be required at different time points during infection and discuss the implication of the cellular distribution of the SpvABCD proteins on their function.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The virulent strain *S. dublin* Lane is a clinical blood isolate that contains the virulence plasmid pSDL2. *S. dublin* LD842 is a pSDL2-cured avirulent isogenic derivative of *S. dublin* Lane (8). *Escherichia coli* DH5α was used as a host for cloning and plasmid constructions (25). The high-copy-number plasmids pUC18 and pUC19 were used for cloning and overexpression of the *spv* genes (25). pTJS124 is a low-copy-number plasmid derived from RK2; it encodes β-lactamase (25, 35). Bacterial cultures were grown in Luria-Bertani broth. When required, penicillin was added to a final concentration of 200 μg/ml. To ensure that the cells were growing exponentially, overnight cultures were diluted 1:100, incubated at 37°C with vigorous shaking (300 rpm) for 2 h, diluted 1:10, and incubated for another 1 h before the assays were started. Samples were removed at the desired optical densities at 600 nm (OD_{600}) or at desired time points.

Immunization procedure and preparation of polyclonal antibodies. The individual *spvA*, *spvB*, *spvC*, and *spvD* genes from pSDL2 of *S. dublin* Lane were

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cloned into the high-copy-number pUC18 and pUC19 plasmids, and each *spv* gene product was overexpressed in *E. coli* DH5α separately. After lysozyme-induced cell lysis, SpvA, SpvB, SpvC, and SpvD were identified in the insoluble cell fraction, indicating that they were packed into inclusion bodies. For solubilization, the insoluble cell fraction was resuspended in 0.1 M Tris buffer (pH 8.5) containing increasing concentrations of urea. SpvA, SpvB, SpvC, and SpvD became solubilized at 1 M, 6 M, 3 M, and 1 M urea, respectively (34). The solubilized Spv proteins were loaded on a preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel stained by the ChromaPhor protein visualization system (Promega). The Spv bands were excised from the gel and eluted in 50 mM ammonium bicarbonate (pH 7.8)–0.1% SDS. New Zealand White rabbits were given subcutaneous injections of 1.0 ml of approximately 0.1 mg of antigen mixed 1:1 with Titer Max adjuvant (Vaxcel, Inc., Atlanta, Ga.). Booster immunizations were carried out after 28 and 56 days. The animals were sacrificed on day 66. To purify the polyclonal antibodies, the antisera were diluted 1:3 with phosphate-buffered saline and dialyzed against 18% Na_2SO_4. The precipitated proteins were dissolved in double-distilled water, dialyzed against TN buffer (50 mM Tris [pH 7.4], 150 mM NaCl), and passed over an Affi-Gel 15 column (Bio-Rad) coupled to the lysate of plasmid-cured *S. dublin* LD842.

DNA manipulations and protein analysis by immunoblotting. Rapid plasmid clone analysis, cleavage with restriction endonucleases, gel purification, ligations, and transformations were performed by standard methods (34). Western immunoblotting was performed as described previously (24). Proteins were separated by SDS-PAGE (10 to 15% polyacrylamide) and transferred electrophoretically to nitrocellulose membranes. The samples were standardized to equal cell amounts or as stated in the text, and they were all prepared under reducing conditions. After they were transferred to nitrocellulose membranes, nonspecific antibody binding was blocked by incubation in 4% (wt/vol) dried milk in Tris-buffered saline (pH 7.5) containing 0.05% Tween 20. The blots were probed with rabbit polyclonal antibodies generated against each of the Spv proteins. Binding of the primary antibody was detected with horseradish peroxidase-labeled second antibodies and enhanced chemiluminescence (ECL) detection reagents (Amersham) followed by exposure to radiographic films. The intensity of the protein bands was quantified by densitometry (no. GS-700, Bio-Rad; Molecular Analyst software program). To construct the figures, the blots were scanned with Adobe Photoshop 3.0 software.

Cell fractionation. A total of approximately 1.5×10^{11} bacterial cells were fractionated into periplasmic, cytoplasmic, inner membrane, and outer membrane components. The periplasmic fraction was prepared as described by Manoil and Beckwith (26) with some modifications. The bacteria were grown to the desired OD_{600}, pelleted, and resuspended in spheroplast solution (100 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.05 mM sucrose). After being mixed on a rotatory shaker for 10 min, the suspension was centrifuged and the pellet was incubated in ice-cold 1 mM $MgCl_2$ for 5 min. After addition of α_2-macroglobulin to a final concentration of 5 μg/ml, the suspension was centrifuged for 10 min at 17,000 × *g*. The supernatant was centrifuged a second time for 30 min at 120,000 × *g* and saved as the periplasmic fraction.

To obtain the cytoplasm, the pelleted cells (see above) were suspended in ice-cold 0.01 mM HEPES (pH 7.5), supplemented with α_2-macroglobulin, and then broken with a French press (36). The unbroken cells were removed by sedimentation at 5,000 × *g* for 5 min at 4°C. To separate the soluble and insoluble components, the broken cells were centrifuged at 200,000 × *g* for 1 h at 4°C. The supernatant was incubated for 30 min at 21°C, centrifuged a second time, and stored on ice as the cytoplasmic fraction. The inner and outer membranes were prepared essentially as described by Schnaitman (36). The pelleted insoluble fraction (see above) was layered over a 55 to 30% (wt/vol) sucrose gradient and centrifuged at 130,000 × *g* for 48 h at 4°C. The inner membrane appeared as the upper band at approximately 35% sucrose, and the outer membrane appeared as a lower band at approximately 50% sucrose. Each band was collected, diluted 1:3 with HEPES buffer containing 1 mM $MgCl_2$ and 5 μg of α_2-macroglobulin per ml, and centrifuged at 200,000 × *g* for 2 h at 4°C. The sedimented membrane particles were then resuspended in equal volumes of HEPES buffer containing α_2-macroglobulin. The final yield of each fraction corresponded to a total of 1.5×10^{11} cells. All assays were performed with fraction samples that stemmed from equal number of bacteria.

Enzyme assays. To determine the purity and cross-contamination of the cytoplasmic, periplasmic, inner membrane, and outer membrane fractions, the activities of the β-lactamase, NADH oxidase, and lactate dehydrogenase (LDH) in the various cell fractions were measured. For each assay, fractions from equal amounts of cells were used. β-Lactamase activity was determined by decolorization of a starch-iodine solution in the presence of penicillin (32). NADH oxidase activity was determined by measuring the oxidation of NADH (30). LDH activity was determined by using the same method as that used for NADH oxidase, except that pyruvate was added to the reaction mixture (38). LDH activity was obtained by subtracting the NADH oxidase activity from the pyruvate-derived activities.

Preparation of culture supernatants. To examine the culture supernatant for the presence of Spv proteins, the bacteria were grown in Luria-Bertani broth. At 1 h prior to collection of the supernatant, the medium was supplemented with α_2-macroglobulin at a final concentration of 5 μg/ml. After centrifugation, the supernatants were passed through a 0.2-μm-pore-size filter to remove the remaining bacteria and supplemented with a protease inhibitor cocktail (complete plus 40 μg of Bestatin per ml [Boehringer]). After an additional centrifugation for 1 h at 130,000 × *g*, the supernatant proteins were concentrated, precipitated, and redissolved in SDS loading buffer for immunoblotting.

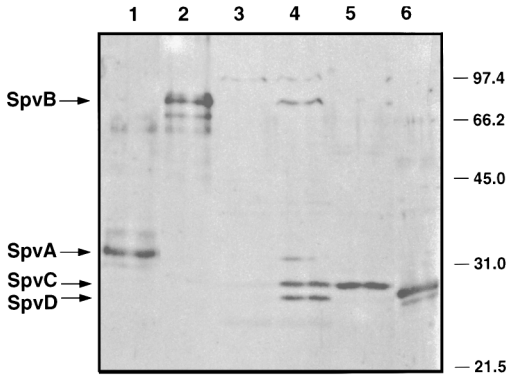

FIG. 1. Identification of the SpvA, SpvB, SpvC, and SpvD proteins in *S. dublin* Lane. Whole cells were examined by Western blotting with pooled anti-SpvA, -SpvB, -SpvC, and -SpvD antibodies. Purified Spv antigens generated in *E. coli* and whole cells of *S. dublin* LD842 were used as positive and negative controls, respectively. Lanes: 1, SpvA antigen; 2, SpvB antigen; 3, *S. dublin* LD842; 4, *S. dublin* Lane; 5, SpvC antigen; 6, SpvD antigen.

Glycoprotein detection. A simple method to detect glycoproteins was applied (5). The reactions were carried out by following the instructions supplied with the ECL glycoprotein detection system (Amersham). Briefly, after SDS-PAGE of whole-cell lysates and transfer to a nitrocellulose membrane, the membrane was treated with sodium metaperiodate to oxidize the carbohydrate groups attached to protein. Then biotin hydrazide was used to incorporate biotin into the oxidized carbohydrates. The biotin was subsequently detected by the addition of streptavidin-horseradish peroxidase conjugate, followed by the addition of ECL detection reagents and exposure of the membrane to radiographic film.

RESULTS

Identification of the SpvABCD proteins in wild-type *S. dublin*. To generate polyclonal antibodies against the virulence proteins SpvA, SpvB, SpvC, and SpvD, we immunized rabbits with the partially purified Spv proteins (see Materials and Methods). Rabbit sera were collected, and the antibody fraction was separated and purified. The presence of specific polyclonal antibodies against SpvA, SpvB, SpvC, and SpvD was confirmed by Western blotting. Each antibody fraction recognized the corresponding Spv antigen generated in *E. coli*, while *E. coli* carrying the vector served as a negative control (data not shown). Next, wild-type *S. dublin* Lane was grown to stationary phase (OD_{600} = 1.8), and whole-cell lysates were tested in comparison with plasmid-cured *S. dublin* LD842. Four distinct bands at 76, 31, 28, and 25 kDa, corresponding to SpvB, SpvA, SpvC, and SpvD, respectively, were identified (Fig. 1). SpvB and SpvA ran at a significantly higher molecular mass than predicted from the nucleotide sequences at 65 and 28 kDa, respectively, suggesting that both proteins undergo further modifications after translation.

Expression profile of SpvABCD. Convincing evidence has been provided that *spv* expression is regulated by stationary-phase growth. We monitored the subcellular SpvA, SpvB, SpvC, and SpvD levels in wild-type *S. dublin* Lane during growth for 25 h (Fig. 2). Synthesis of the Spv proteins was initiated in the late exponential phase. They were detectable in the sequence SpvA, SpvB, SpvC, and SpvD at OD_{600}s of 0.50, 0.67, 1.0, and 1.1, respectively. Both SpvA and SpvB reached their maximum at an OD_{600} of approximately 1.5 to 1.8, and their levels decreased substantially thereafter. In contrast, the levels of SpvC increased continuously and those of SpvD remained high until late stationary phase.

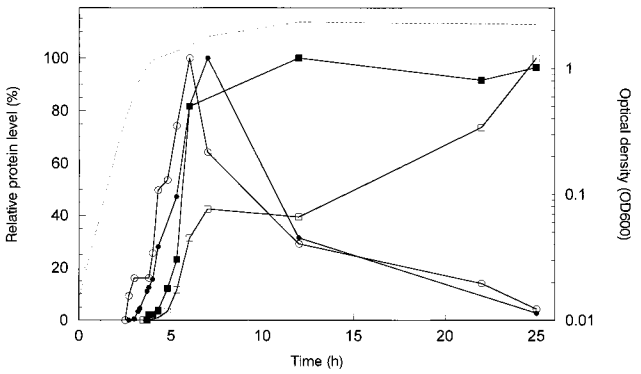

FIG. 2. Expression profile of the SpvA, SpvB, SpvC, and SpvD proteins in *S. dublin* Lane during growth. Open circles, SpvA; solid circles, SpvB; open squares, SpvC; solid squares, SpvD; solid lines, Spv levels; dotted line, growth curve (OD_{600}). The relative levels were plotted.

Previous mRNA studies and analysis by *spv*::*lacZ* reporter gene expression indicated that the individual *spv* genes are expressed in decreasing abundance in the order SpvA > SpvB > SpvC > SpvD (23, 27). However, we consistently observed a different pattern of the cellular Spv concentrations in *S. dublin* (Fig. 1). It was particularly striking that low SpvA and high SpvD levels contrasted with the abundance of *spvA* mRNA and the hardly detectable *spvD* mRNA signals that we found previously (23). This could be due simply to differences in the avidity of the individual anti-Spv antibodies to their antigens, however, since standardized amounts of purified SpvA, SpvB, SpvC, and SpvD were tested and the qualities of the four antibody fractions were comparable.

Fractionation and determination of the purity of the bacterial cell components. To determine the subcellular location of the SpvA, SpvB, SpvC, and SpvD proteins in wild-type *S. dublin* Lane, the cells were grown to an OD_{600} of 1.8 and fractionated into the periplasmic, cytoplasmic, outer membrane, and inner membrane fractions as outlined in Materials and Methods. The cellular fractions were identified by SDS-PAGE (Fig. 3). The purity was assessed by measuring the activities of marker enzymes specific for each fraction with samples from approximately 2×10^7 bacterial cells. The β-lactamase is located exclusively in the periplasm, the NADH oxidase is located in the inner membrane, and LDH is located in the cytoplasm. Since *S. dublin* Lane and LD842 do not produce β-lactamase, pTJS124, a low-copy-number RK2 derivative carrying a β-lactamase gene, was introduced into LD842 (25). The contamination with outer membrane components was assessed by the presence of the *S. dublin* major outer membrane proteins on SDS-PAGE (Fig. 3). The purity of the single fractions based on the enzyme activity assays is shown in Table 1. The yield of the periplasmic component was low, as indicated by its containing only 16% of the total β-lactamase activity. It contained <1% of the total LDH activity, ensuring that the fraction was not contaminated by the cytoplasm; also, no contamination by the inner or outer membranes was detectable. The cytoplasm contained 99% of the total LDH activity; however, the large portion of the total β-lactamase activity indicated that most of the periplasm was contained in the cytoplasm. Less than 10% of the total NADH oxidase activity was detectable in the cytoplasmic fraction, indicating a low level of contamination by the membranes. The membrane components were free of contamination by the periplasm or by the cytosol. Also, cross-contamination between the inner and outer membranes was low.

TABLE 1. The relative activities of the marker enzymes β-lactamase, LDH, and NADH oxidase in the cell fractions

Marker enzyme	% Enzyme activity in[a]:				
	Periplasm	Cytoplasm	Insoluble fraction	Inner membrane	Outer membrane
β-Lactamase	16	84	<1		
LDH	<1	99	<		
NADH oxidase	<1	9	90	81[b]	9[b]

[a] The total of the enzyme activities in the periplasmic, cytoplasmic, and insoluble fractions was considered as 100%.
[b] The activities of NADH oxidase in the inner and outer membrane fractions were related to the activity in the insoluble fraction.

Subcellular location of the SpvABCD proteins. Next, we evaluated the subcellular location of the SpvABCD proteins in fractionated *S. dublin* Lane by immunoblotting. The corresponding fractions of *S. dublin* LD842 served as negative controls. To compare the fractions, it was important to load samples adjusted to equal numbers of cells on the gels. Also, to compensate for the low yield of the periplasm, an approximately 10-fold amount of this fraction was used. None of the Spv proteins were detected in the periplasm. SpvA was identified exclusively in the outer membrane fraction (Fig. 4A). SpvB was detected in both the inner membrane fraction and the soluble component; however, the inner membrane portion was 5- to 10-fold less abundant than the portion in the soluble cell component (Fig. 4B). SpvC was detected exclusively in the soluble component, indicating that it is a pure cytosolic protein (Fig. 4C). SpvD was detected in all cell fractions except the periplasm. The SpvD concentration in the inner membrane was relatively low compared with the concentrations in the soluble and outer membrane fractions (Fig. 4D).

Exported proteins may become glycosylated by being passed through the periplasm. We did not detect glycoproteins at 31 and 25 kDa that would correspond to SpvA and SpvD, respec-

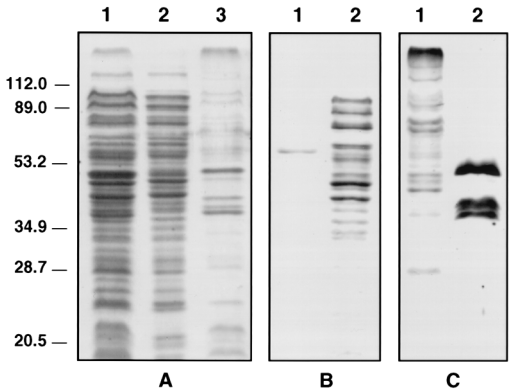

FIG. 3. Cell fractions of *S. dublin* Lane subjected to SDS-PAGE (12% polyacrylamide) and stained with Coomassie brilliant blue. (A) Bacterial cells were broken and centrifuged to separate the soluble and insoluble components. Lanes: 1, broken whole cells; 2, soluble fraction (cytoplasm plus periplasm); 3, insoluble fraction (membranes). (B) The cells were subjected to osmotic shock to obtain the periplasm and were then broken to release the cytoplasm. Lanes: 1, periplasmic fraction (17-fold concentrated); 2, cytoplasmic fraction. (C) The insoluble fraction was separated by sucrose density gradient centrifugation into the inner and outer membranes. Lanes: 1, inner membrane fraction; 2, outer membrane fraction. The components loaded in panels A, B, and C do not correspond to equal bacterial numbers.

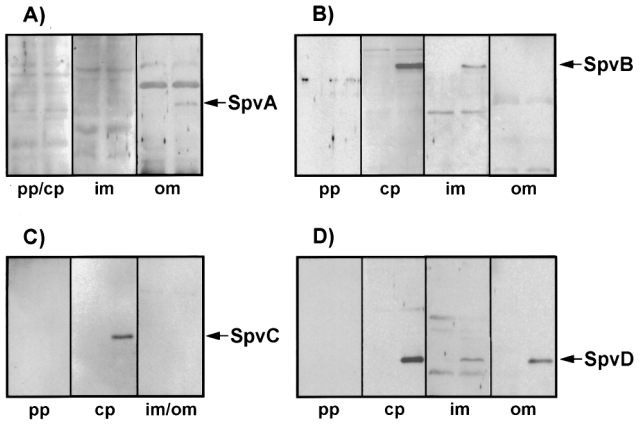

FIG. 4. Localization of the virulence proteins SpvA, SpvB, SpvC, and SpvD in *S. dublin* Lane. Panels A to D were probed with antibodies against the proteins SpvA, SpvB, SpvC, and SpvD, respectively. Left lanes, cellular fractions of *S. dublin* LD842 (negative control); right lanes, cellular fractions of *S. dublin* Lane. pp, periplasm; cp, cytoplasm; im, inner membranes; om, outer membranes; pp/cp, soluble fraction (cytoplasm plus periplasm); im/om, insoluble fraction (inner plus outer membranes).

tively. However, two glycoprotein bands at 22 and 22.5 kDa were found in *S. dublin* Lane that were not present in *S. dublin* LD842, indicating that a plasmid locus apart from the *spv* region encodes two proteins that become glycolyzed (data not shown).

SpvD is secreted in culture supernatants. To address the question whether any of the structural proteins were exported out of the cell, we examined the supernatants of *S. dublin* Lane at the time points when the cellular concentration of each Spv protein was at its maximum (Fig. 2). The culture supernatants were prepared as outlined in Materials and Methods and examined by Western blotting with the specific polyclonal anti-SpvABCD antibodies. Only SpvD was detectable, indicating that it was the only secreted Spv protein (Fig. 5). SpvD was easily detectable in the supernatant of stationary-phase cultures but was not detectable during earlier stages of growth or when protease inhibitors were not added.

DISCUSSION

In most of the previous studies, *spvABCD* expression was investigated by using cloned *spv*::*lacZ* reporter fusion constructs (2, 7, 10, 21, 22, 27, 37). Although these studies have provided a profound understanding of the transcriptional regulation of the *spv* genes, the determination of β-galactosidase activities from fusion proteins may have some disadvantages. First, most of the cloning vehicles carrying the *lacZ* fusions used in these studies replicate at higher copy numbers than the wild-type plasmid, which might disrupt the stoichiometric control between chromosomal and plasmid-derived virulence factors. Second, interruption of the *spv* operon by *lacZ* insertions may have polar effects on the expression of the downstream genes and may disturb proper interactions among the individual Spv proteins. Third, the Spv-LacZ fusion proteins may impede proper localization and may have degradation kinetics different from those of the wild-type Spv proteins. To avoid these drawbacks, we used polyclonal anti-Spv antibodies to examine the kinetics of SpvA, SpvB, SpvC, and SpvD in wild-type *S. dublin* during growth. Surprisingly, only one study investigated the expression of the SpvA, SpvB, and SpvC proteins from the native plasmid in more detail (44). In that study, a variety of environmental stress conditions that induce Spv expression were defined. Another laboratory tested SpvA expression by Western blotting in *S. typhimurium* and *S. dublin* during stationary-phase growth (40). A weak SpvA band was obtained in *S. dublin*, whereas SpvA was not detectable in *S. typhimurium.*

In accordance with the regulation of *spvABCD* transcription, we found that Spv protein production is initiated in late exponential growth. Furthermore, the proteins were detectable in the sequence SpvA, SpvB, SpvC, and SpvD, which was also the sequence of appearance of the specific *spv* mRNAs in wild-type *S. dublin* (23). During further growth, however, the cellular concentrations of the individual SpvABCD proteins showed different courses. Both SpvA and SpvB concentrations reached a maximum in early stationary phase and declined significantly thereafter. This finding was not consistent with the data obtained from mRNA analysis and the *spv*::*lacZ* fusions studies, which indicated that the highest levels of expression of *spvA* and *spvB* occur in the late stationary phase. SpvC and SpvD levels, however, continued to increase or remained high throughout stationary-phase growth, respectively, which was in agreement with the earlier reports. A certain portion of SpvD is secreted into the supernatant; therefore, the net SpvD production during growth remains unknown. Since *spvABCD* are transcribed from the same promoter, it is conceivable that distinct posttranscriptional regulations at the level of translation or protein stability are responsible for the divergent Spv levels, although experimental evidence for this is lacking. Another remarkable observation was that the maximal SpvA concentrations were substantially lower than the maximal SpvD concentrations, since at the level of transcription, the specific *spvA* and *spvD* mRNA species gave a ratio of 30:1 (23). Differences in the qualities of the individual antibodies may be at least partially responsible for this finding; however, low-level SpvA signals were also found by another group (40), supporting our hypothesis that posttranscriptional processing may account for the discrepancy between mRNA and protein concentrations.

In an earlier report, the cellular locations of SpvA, SpvB, and SpvC were determined in *S. typhimurium* carrying the cloned *spvABC* genes behind an exogenous promoter on a high-copy-number vector (43). Unfortunately, protein overproduction frequently results in nonphysiological compartmentalization, including the formation of inclusion bodies, which brings into question the significance of these data. In this work, we provide the first data about the subcellular distribution of the SpvABCD proteins in a wild-type *Salmonella* strain. We fractionated wild-type *S. dublin* into the periplasmic, cyto-

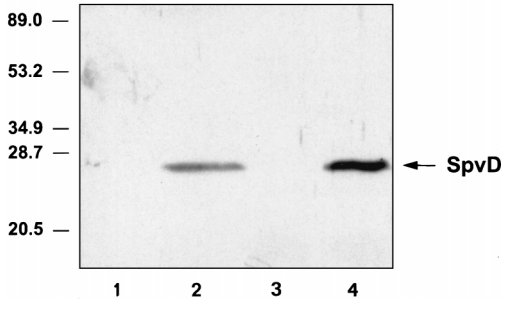

FIG. 5. Detection of the virulence protein SpvD in supernatants of *S. dublin* Lane LB cultures (OD_{600} = 2.3). The blot was probed with anti-SpvD antibodies. Lanes: 1, supernatants from *S. dublin* LD842 (control); 2, supernatant from *S. dublin* Lane; 3, whole cells of *S. dublin* LD842 (control); 4, whole cells of *S. dublin* Lane. The amount of secreted SpvD in lane 2 is produced within 1 h by a cell number 10-fold greater than that loaded in lane 4.

solic, and membrane components and separated the inner and outer membranes by sucrose gradient centrifugation. The fractions were analyzed by immunoblotting with specific polyclonal antibodies.

SpvA was found exclusively in the outer membrane. The identity of the outer membrane component was confirmed by the predominance of the characteristic major outer membrane proteins on SDS-PAGE. The purity was assessed by the criterion of less than 1% activity for the cytoplasmic and periplasmic markers and less than 10% for the inner membrane enzyme marker. The translocation of SpvA is supported by an earlier report that mapped numerous Tn*PhoA* transposon mutations within the *spvA* gene, which exhibited alkaline phosphatase activity (43). To get through the cytoplasmic membrane, SpvA appears to use a mechanism other than the general secretory pathway, since it does not fulfill all the postulated characteristics of a typical bacterial signal peptide, although it carries a strongly hydrophobic N terminus (25). This export mechanism seems to be highly efficient, since SpvA is also found exclusively in the outer membrane when it is overproduced from an exogenous promoter (43).

SpvB was found in two fractions. The major portion of SpvB was found in the cytoplasmic fraction, and a smaller one was found in the inner membrane fraction. One explanation for this is that SpvB is weakly associated with the inner membrane and that it became solubilized in the cytoplasmic component during cell fractionation. The primary structure of SpvB is remarkable for having a long run of prolines (nine in *S. dublin*, seven in *S. typhimurium*) that may serve to separate the higher-order structure of the protein into different domains, for example, into cytoplasmic and membrane domains.

SpvC accumulated exclusively within the cytoplasmic fraction. Due to significant cross-contamination of the cytoplasm with the periplasm, it was important to establish that SpvC did not stem from the periplasm. Since the periplasmic fraction was free of cytoplasm and SpvC was not detectable in that fraction, SpvC is a cytoplasmic virulence protein.

SpvD is exported out of the cell into the supernatant. In the supernatant, this protein was detectable only in the presence of proteinase inhibitors, suggesting a rapid degradation in this milieu. Interestingly, significant amounts of SpvD were also found in the cytosolic and both the inner and outer membrane fractions (Fig. 4), a situation similar to that described for the Ipa proteins from in vitro-cultured *Shigella* (3, 28). The large cell-associated pool of SpvD may result from a relatively inefficient excretion process and a simultaneous high SpvD turnover rate due to the high susceptibility of extracellular SpvD to proteolysis. SpvD carries a hydrophilic N terminus and is not cleaved during export into the extracellular milieu, since both cellular SpvD and secreted SpvD run at the predicted size of 25 kDa on SDS-PAGE, strongly indicating that it is exported by an alternative secretory mechanism. One possibility is that SpvD is secreted by the type III protein secretion system that has recently been recognized in a variety of enteric pathogens, including *Shigella*, *Yersinia*, *E. coli*, and *Salmonella*, and that is characterized by secretion of target proteins without cleavage (45).

Knowledge of the distribution of the Spv proteins may give important clues about their functions. SpvA has been proposed to have a negative regulatory function for *spvR* expression (1). Such a role is conceivable before SpvA leaves the cytoplasm; however, after SpvA is exported, it appears to take over another function. Surprisingly, *spvA* mutants do not reduce the virulence of *S. dublin* in mice infected by the peritoneal route, bringing into question the importance of SpvA. However, its role in oral infections and in the native host remains to be determined. SpvB is linked to the cytoplasmic membrane, suggesting a transmembrane-associated function. It shows some homologies to the Ace protein of *V. cholerae*, which is capable of altering cellular ion fluxes; however, experimental evidence for a similar function of SpvB is lacking (42). *spvB* mutants are completely avirulent, indicating a pivotal role of SpvB in the plasmid-encoded virulence phenotype (33). It is remarkable that SpvB and SpvC remain intracellular, which indicates that they both express virulence by mechanism other than by a direct interaction with the host factors. The bacterial growth kinetics in mouse models of salmonellosis suggest that the *spv* genes confer an increased growth rate within host cells (19, 20), and *spv* expression is dramatically induced by the intracellular milieu of eukaryotic cells (13, 31). Hypothetically, SpvB and SpvC could enable the bacterial cell to replicate more rapidly in the intracellular intraphagosomal environment, where bacterial growth is normally restricted by a limited supply of essential nutrients. SpvD is the fourth virulence product of the *spvABCD* operon and has gained little attention in previous reports, although its role in virulence has been established in mice (19, 25, 33). The evidence that SpvD is exported raises the question whether it exerts toxic effects on host cells; however, experimental data to support this are lacking.

In summary, we have determined the expression profile of the individual SpvABCD proteins during growth and found them to be localized in different compartments of the bacterial cell. Our study provides the basis for future studies that should be directed toward the function rather than toward the regulation of expression of the *spvABCD* operon. It is conceivable that the four Spv proteins may have related activities and fulfill complex enzymatic and/or structural functions that require a coordinate action. These activities might be required at different time points during the infection, since the proteins appear and peak in a specific timely sequence.

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