

Virulence of a *Salmonella typhimurium* OmpD Mutant

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Received 31 July 1997/Returned for modification 23 September 1997/Accepted 23 October 1997

An *ompD* mutation caused by a *Tn10* insertion was transduced into *Salmonella typhimurium* SL1344 and UK-1. The adherence and invasion capabilities of the resultant *ompD* mutants were examined by tissue culture analysis. The virulence of the *S. typhimurium ompD* mutants was ascertained by a 50% lethal dose (LD₅₀) study and by determining colonization ability with BALB/c mice. We found no statistically significant difference in adherence and invasion capacities between the *S. typhimurium* wild type strains and their corresponding *ompD* mutants. Furthermore, the LD₅₀ and colonization studies revealed that there is no statistically significant difference in virulence between the *S. typhimurium* wild type strains and their corresponding *ompD* mutants. These results differ from those reported previously (C. J. Dorman, S. Chatfield, C. F. Higgins, C. Hayward, and G. Dougan, *Infect. Immun.* 57:2136–2140, 1989).

Salmonella enterica serovar Typhimurium, a gram-negative bacterial species, is a facultative intracellular pathogen which infects its hosts through the oral route (25). Human diseases caused by *Salmonella* serotypes include gastroenteritis, bacteremia, and typhoid fever (11). Most infections occur as a result of ingestion of undercooked eggs or contaminated food (meats and dairy products) or water (1, 11, 22). Each year in the United States two to four million cases of gastroenteritis are caused by *Salmonella* bacteria (23), along with a few hundred cases of typhoid fever (3). According to the World Health Organization, *Salmonella* is probably the most common cause of diarrhea globally (11), and at least 12 million cases of typhoid fever are reported each year, with a mortality rate of 10 to 12% (7).

Like other gram-negative bacteria, *S. typhimurium* has an outer membrane surrounding the periplasmic space. The outer membrane contains numerous proteins, referred to as OMPs. A subset of these, called porins, form water-filled channels across the outer membrane to facilitate the transport of small hydrophilic molecules (16). *S. typhimurium* expresses three porins when grown under normal conditions (Lennox broth at 37°C): OmpD (34 kDa), OmpF (35 kDa), and OmpC (36 kDa) (12, 15, 21). OmpD is found in *S. typhimurium* but is absent from other gram-negative bacteria, including *Escherichia coli*. OmpD is homologous with the NmpC and Lc porins in *E. coli* K-12 (21), both of which (NmpC and Lc) can only be expressed in *E. coli* K-12 mutants which lack normal outer membrane proteins (18). Little is known about the OmpD porin, apart from the genomic location of the *ompD* gene and the immunochemical and topological structure of the porin itself (20, 21).

Dorman and colleagues (6) showed that mutations in some porin-associated genes affect the virulence of *S. typhimurium* in BALB/c mice. Specifically, a mutation in the *ompR* gene, which encodes a positive regulator of porin gene expression, has a dramatic effect on virulence, increasing the 50% lethal dose (LD₅₀) by more than three log units compared to that of a wild-type strain. In the same study, Dorman et al. character-

ized the effect of mutations in the *ompC*, *ompF*, and *ompD* genes. They report that strains containing *ompF* or *ompC* mutations were as virulent as their wild-type parent, while a strain containing an *ompD* mutation showed a slight reduction in virulence (23-fold increase in LD₅₀ between the wild type and the *ompD* mutant). Interestingly, OmpR regulates the expression of the genes coding for porins OmpC and OmpF; but it does not seem to regulate expression of *ompD* (6).

In a subsequent study, Chatfield et al. (2) showed that a mutant lacking both the OmpF and OmpC porins is attenuated, displaying an oral LD₅₀ that is three log units greater than that of the wild-type parent. This result explains, in part, the attenuation of *ompR* mutants. However, because *ompR* mutants display higher oral and intravenous LD₅₀s than the *ompF ompC* double mutant, it is likely that there are other genes regulated by OmpR which encode proteins involved in virulence.

Traditional programs to design live, attenuated oral vaccines against *Salmonella* have concentrated on using mutations in the bacterial biochemical pathways or using mutations in global regulators (19). Eliminating global regulators can render a strain avirulent and immunogenic. Inactivating some of the genes regulated by a global regulator should account for some of the avirulence and immunogenicity seen in strains containing a mutation in the global regulator. The *ompD* gene is regulated by adenylate cyclase and the cyclic AMP regulatory protein (CRP) (16). Strains of *S. typhimurium* which have *cya* and *crp* mutations are avirulent (4). Our goal was to determine if a mutation in *ompD* could account for some of the avirulence of the *cya* and *crp* mutants. In this study, an *ompD* mutation was transduced into virulent *S. typhimurium* SL1344 and UK-1. The resultant transductants were compared with their wild-type parents with respect to their abilities to adhere to and invade cells in culture and to colonize tissues and cause disease in BALB/c mice. In contrast to the results of a previous study (6), our results show that *ompD* mutants are not attenuated, eliminating the possibility that nonexpression of OmpD contributes to the avirulence of *cya* and *crp* mutants.

Bacterial strains, media, and phenotypic screens. The bacterial strains used in this study are listed in Table 1. To generate strains specifically for this project, standard P22HTint transductions were performed. Strains were constructed by transducing the *ompD::Tn10* mutation from strain BRD455 (6) into virulent *S. typhimurium* SL1344 strain χ 3339 (9) and UK-1

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TABLE 1. Bacterial strains

Strain	Genotype	Source (reference)
<i>S. typhimurium</i> SL1344 χ 3339	<i>rpsL hisG</i>	This laboratory (9)
BRD454	<i>rpsL hisG ompC396::Tn10</i>	Dorman et al. (6)
BRD455	<i>rpsL hisG ompD159::Tn10</i>	Dorman et al. (6)
χ 3643	<i>rpsL hisG invA::TnphoA</i>	Galán and Curtiss (8)
χ 8201	<i>rpsL hisG ompD159::Tn10</i>	This study
<i>S. typhimurium</i> UK-1 χ 3761	Wild type	This laboratory (5)
χ 8202	<i>ompD159::Tn10</i>	This study

strain χ 3761 (5) to yield strains χ 8201 and χ 8202, respectively. Transductants were purified, and the presence of the *ompD* mutation was verified by examining outer membrane fractions from χ 8201 and χ 8202 by protein electrophoresis as described below.

The standard media used for this study were Lennox broth (13), Luria-Bertani broth (14), and MacConkey agar (Difco). Lennox broth was supplemented with 1.5% agar for plates. MacConkey agar was supplemented with lactose at 1.0%. Tetracycline (Gibco BRL, Grand Island, N.Y.) was added to Lennox broth and MacConkey agar at a concentration of 15 μ g/ml.

Membrane isolation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Bacterial cells were grown in Lennox broth overnight and then sedimented by centrifugation at 5,000 rpm (Sorvall SS-34 rotor in a Sorvall RC5C centrifuge) at 4°C for 10 min and resuspended in phosphate-buffered saline at pH 7.4. Cells were lysed with a French press at 2,000 psi. After the cellular debris was removed by centrifugation at 6,000 rpm (Sorvall SS-34 rotor in a Sorvall RC5C centrifuge) at 4°C for 10 min, the outer membrane proteins were specifically selected by ultracentrifugation at 36,000 rpm (Sorvall SW41Ti rotor in a Sorvall OTD65B ultracentrifuge) at 4°C for 1 h. The pellet containing both cytoplasmic and outer membrane proteins was resuspended in phosphate-buffered saline containing 0.5% Sarkosyl (sodium lauryl sarcosinate) (Sigma, St. Louis, Mo.), followed by another round of ultracentrifugation at 36,000 rpm at 4°C for 1 h to precipitate the outer membrane fractions. Tris-glycine SDS-polyacrylamide gels (10% acrylamide and 1.5 M Tris [pH 8.8] for the slab gel; 5% acrylamide and 1.0 M Tris [pH 6.8] for the stacking gel) were used to separate the outer membrane proteins. The gels were stained with 0.25% Coomassie blue stain and destained with a solution of 10% glacial acetic acid and 30% methanol.

Virulence assays. The abilities of *S. typhimurium* mutants to adhere to and invade Intestine-407 (Int-407) cells (10) were analyzed by using a protocol based on a method developed by Galán and Curtiss (8), as described previously (24).

Seven- to ten-week-old female BALB/c mice were used for all animal experiments. The mice were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and kept at least 1 week prior to inoculation. Virulence was assayed by a comparison of the LD₅₀s of wild-type and mutant strains and by a comparison of the abilities of wild-type and mutant strains to colonize various tissues at 1, 3, and 6 days postinfection.

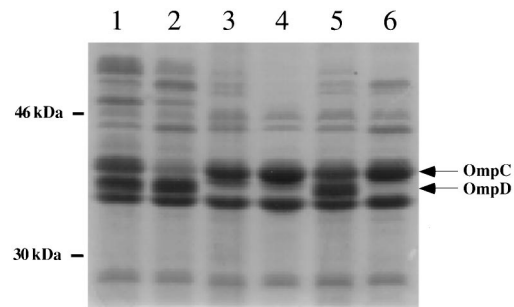


FIG. 1. Separation of outer membrane fractions of six strains of *S. typhimurium* by SDS-10% PAGE. Lane 1, wild-type SL1344 strain χ 3339; lane 2, *ompC* mutant BRD454; lane 3, *ompD* mutant BRD455; lane 4, *ompD* mutant SL1344 strain χ 8201; lane 5, wild-type UK-1 strain χ 3761; lane 6, *ompD* mutant UK-1 strain χ 8202.

For the LD₅₀ experiment, strains were grown in Luria-Bertani broth overnight and then subcultured at a 1:200 dilution and grown to an optical density at 600 nm of between 0.7 and 1.0. The cells were concentrated by centrifugation and resuspended in buffered saline with gelatin (BSG), after which dilutions were made in BSG to obtain three different doses for each strain. At each dose, four mice were given oral inoculations of 20 μ l of *S. typhimurium* suspension per mouse. The mice were observed for a period of 4 weeks. LD₅₀s were calculated by the method of Reed and Meunch (17).

For the colonization experiment, bacteria were grown as described above and concentrated in BSG approximately 10-fold. The wild-type and mutant suspensions were mixed to give a ratio of mutant/wild-type bacteria of approximately 1.0. Twelve mice were each infected perorally with 20 μ l of the bacterial suspension after being deprived of food and water for 4 to 6 h. Food and water were returned 30 min after infection. At 1, 3, and 6 days postinoculation, four mice were euthanized and the Peyer's patches, intestinal wall, intestinal contents, spleen, and liver were removed from each mouse. Each tissue was placed in 2 ml of cold BSG and homogenized with a Brinkmann homogenizer. The bacteria were enumerated after the dilutions were plated on MacConkey lactose agar as well as MacConkey lactose agar plus tetracycline.

Verification of the *ompD* mutants. As described above, the *ompD::Tn10* mutation was transduced into *S. typhimurium* SL1344 strain χ 3339 and *S. typhimurium* UK-1 strain χ 3761 to obtain χ 8201 and χ 8202, respectively. SDS-PAGE analysis of outer membrane fractions from χ 8201 and χ 8202 was performed to verify the absence of the protein band corresponding to OmpD. The results confirm that both χ 8201 and χ 8202 strains did not express OmpD (Fig. 1).

Adherence and invasion capabilities of the *ompD* *S. typhimurium* mutants. Dorman and colleagues (6) reported that a mutation in *ompD* had only a small effect on the virulence of *S. typhimurium* SL1344 when BALB/c mice were inoculated perorally. Consequently, the abilities of *ompD* mutants to adhere to and invade intestinal epithelial cells were compared with those of the wild type. The effect of the *ompD* mutation was examined in both the virulent SL1344 and UK-1 backgrounds. As shown in Table 2, in both SL1344 and UK-1 backgrounds, the *ompD* mutants show no significant difference from the wild type in either adherence to or invasion of Int-407 cells. This result indicates that the OmpD porin is not involved in either the adherence or invasiveness of *S. typhimurium*.

Determination of the virulence of wild-type and *ompD* strains of *S. typhimurium* with BALB/c mice. As our results

TABLE 2. Adherence to and invasion of Int-407 cells by wild-type, *ompD*, and *invA* strains of *S. typhimurium*

Strain	% Adherence (<i>P</i> value ^a)	% Invasion (<i>P</i> value ^a)
χ3339	37.7 ± 3.4	15.8 ± 8.8
χ8201	40.4 ± 14.4 (NS)	25.4 ± 14.9 (NS)
χ3643	27.0 ± 6.9 (NS)	0.7 ± 0.4 (≤0.025)
χ3761	41.6 ± 11.5	7.0 ± 3.8
χ8202	39.8 ± 4.9 (NS)	9.5 ± 4.9 (NS)

^a *P* values are given only when the percent adherence or invasion for a mutant is significantly different from that of its wild-type parent (NS, not significant). The test statistic was calculated from the following equation: $t = (\bar{y}_1 - \bar{y}_2)/s \sqrt{[(1/n_1) + (1/n_2)]^{1/2}}$. \bar{y}_1 is the mean for percent adherence or percent invasion for wild type; \bar{y}_2 is the mean for percent adherence or percent invasion for each mutant strain; *s* is the standard deviation; *n*₁ is the number of samples averaged in \bar{y}_1 ; *n*₂ is the number of samples averaged in \bar{y}_2 .

showed no effect of the *ompD* mutation on adherence or invasion, we decided to assay virulence in an animal model. The LD₅₀s of the *ompD* mutants and their corresponding wild-type parents were determined. This was done to verify the original observation made by Dorman et al. (6) that *ompD* mutants were somewhat attenuated. Concurrently, we performed colonization studies to determine at which point in the infection process these mutants may be blocked.

The peroral LD₅₀s for wild-type strains and *ompD* mutants are 1.2×10^5 for strain χ3339 (SL1344), 7.5×10^4 for strain χ8201 (SL1344 *ompD*::Tn10), 3.1×10^5 for strain χ3761 (UK-1), and 1.1×10^5 for strain χ8202 (UK-1 *ompD*::Tn10). In our study, the *ompD*::Tn10 mutation did not increase the LD₅₀ in either background. In fact, the LD₅₀s are slightly lower for the *ompD* mutants.

For colonization studies, mice were coinfectd with an *ompD* mutant and its respective wild-type parent. Representative data from one of the colonization studies are presented in Table 3. Colonization experiments were conducted twice with *S. typhimurium* SL1344 and three times with *S. typhimurium*

TABLE 3. Statistical analysis of a colonization study of *S. typhimurium* SL1344 and an *ompD* derivative by using a paired difference test^a

Time postinoculation (days)	Tissue	Mean paired difference ^b	<i>P</i> value ^c
3	Peyer's patches	-1.1 ± 0.7	NS
	Intestinal wall	-0.6 ± 0.8	NS
	Intestinal contents	0.2 ± 0.3	NS
	Spleen	0.9 ± 2.4	NS
	Liver	-0.2 ± 1.3	NS
6	Peyer's patches	-1.3 ± 1.0	NS
	Intestinal wall	-1.1 ± 0.6	NS
	Intestinal contents	-1.3 ± 1.3	NS
	Spleen	-0.1 ± 0.1	NS
	Liver	-0.4 ± 0.2	NS

^a Mice were inoculated with a mixed suspension of bacteria at a ratio of strain χ3339 cells/χ8201 cells of 1.5.

^b Mean of the paired difference (log₁₀ CFU of strain χ3339 - log₁₀ CFU of strain χ8201) ± the standard deviation.

^c *P* values are given only when significant (NS, not significant). The test statistic (*t*) was calculated as follows: $t = (d - \mu_0)/s_d/n^{1/2}$ where *d* is the mean paired difference, μ_0 is the difference of the inoculum (log₁₀ CFU of strain χ3339 - log₁₀ CFU of strain χ8201 [0.18]), *s*_{*d*} is the standard deviation, and *n* is the sample size (the sample size was four mice, except in a few cases where four mice were tested but results were obtained only for two or three mice).

UK-1. Our studies indicate that there is no consistently significant difference between the *ompD* mutants and their respective wild-type parents in their abilities to colonize either the Peyer's patches, intestinal wall, intestinal contents, spleen, or liver at any of the time points. An occasional increase was seen in either an *ompD* mutant's or its parent's ability to colonize a tissue; however, these differences were not consistent or repeatable.

Discussion. Dorman and colleagues (6) have reported that an *ompD* mutant of *S. typhimurium* SL1344 is less virulent than the wild type, with an oral LD₅₀ about 23-fold higher. LD₅₀s determined in this study show that there is no difference in virulence between *ompD* mutants of *S. typhimurium* SL1344 or UK-1 and their corresponding wild-type parents. In support of this conclusion, our colonization data show that there is no significant difference between the abilities of two different wild-type *S. typhimurium* strains and their respective *ompD* mutants to colonize or reach the Peyer's patches, intestinal wall, intestinal contents, spleen, or liver. Furthermore, adherence and invasion assays performed with cultured intestinal epithelial cells showed no significant difference between the wild type and *ompD* mutants of either *S. typhimurium* UK-1 or SL1344 in their abilities to adhere to or invade host cells.

Why are the results from our study different from those obtained by Dorman et al. (6)? It is possible that differences exist in the BALB/c mice used in the two studies. For example, there may be mild genetic differences between the different mouse colonies which affect their susceptibilities to *S. typhimurium*. Alternatively, the BALB/c mice used by Dorman et al. may have had an additional infection, possibly compromising their ability to recover from a *Salmonella* infection. Another possibility is that subtle differences exist in the manner in which the mice were infected or cared for and that these may account for the differences observed.

The differences in experimental results are unlikely to be due to the nature of the mutations tested, as the particular *ompD*::Tn10 insertion used in this study was the same as that described previously (6). However, the specific *ompD* strain used by Dorman et al. (6) may have acquired an additional mutation during construction which could be the actual cause of the decrease in virulence. It is possible that when the OmpD porin is eliminated by mutation, another may function in its place; hence, a "backup system" may exist. The fact that an *ompC* mutant or an *ompF* mutant (either of which still synthesizes the two other porins) is still virulent (6) could support this hypothesis. If the *ompD* mutant used by Dorman et al. (6) had an additional mutation in this backup system, the mutation may account for the strain's slight attenuation.

Support for this study was provided by a grant to Washington University from the Howard Hughes Medical Institute through the Undergraduate Biological Sciences Education Program and by a grant from the National Institute of Allergy and Infectious Diseases.

We thank Lisa Burns-Keliher for help in the preparation of Fig. 1 and Cheryl Nickerson for helpful discussion.

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Editor: P. E. Orndorff