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Characterization of Porin and *ompR* Mutants of a Virulent Strain of *Salmonella typhimurium*: *ompR* Mutants Are Attenuated In Vivo

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The *ompC*, *ompD*, and *ompF* genes encode the three major porins of *Salmonella typhimurium*. *ompR* encodes a positive regulator required for the expression of *ompC* and *ompF*. Transposon-generated mutations in *ompC*, *ompD*, *ompF*, and *ompR* were introduced into the *S. typhimurium* mouse virulent strain SL1344 by P22-mediated transduction. Following preliminary characterization in vitro, the strains were used to challenge BALB/c mice by using the oral or intravenous route. Strains harboring *ompC* or *ompF* mutations were as virulent as SL1344 after oral challenge. Strains harboring *ompD* mutations had a slight reduction in virulence. In contrast, *ompR* mutants failed to kill BALB/c mice after oral challenge and the intravenous 50% lethal dose was reduced by approximately 10^5. The *ompR* mutants persisted in murine tissues for several weeks following oral or intravenous challenge. Furthermore, mice orally immunized with these *ompR* mutant strains were well protected against challenge with virulent SL1344.

The *ompR* and *envZ* genes form a two-gene operon, previously designated *ompB*. The nucleotide sequence of this locus is highly conserved between *Escherichia coli* and *Salmonella typhimurium* (13, 24). OmpR has been identified as a positive activator of gene expression (20, 39), while EnvZ is thought to be associated with the inner membrane (11). It has been proposed that EnvZ acts as an environmental sensor and transmits signals to OmpR, which then modulates transcription of various genes (14). A pleiotropic mutation in *envZ* has been shown to be suppressed by a mutation in *ompR*, providing genetic evidence that the products of these genes interact functionally (29). Moreover, supressor mutations of *envZ* have been mapped to *rpoA*, the gene coding for the A subunit of RNA polymerase. This suggests that OmpR and RNA polymerase may also interact (12, 28).

OmpR-dependent genes include those coding for the major outer membrane porins, OmpC and OmpF, although OmpD expression seems to be OmpR independent. In addition, *ompR* has been reported as regulating the *S. typhimurium* *tppB* locus, which codes for a tripeptide permease (13), and the *E. coli* genes coding for microcin B17 (15), as well as other uncharacterized genes (13). Binding sites for the OmpR protein have been biochemically identified upstream of the *ompC* and *ompF* promoters (39). Expression of the two porins is reciprocally regulated by growth medium osmolarity in an OmpR-dependent manner (1, 20, 35, 51). In growth media of high osmolarity, the level of OmpC is elevated while that of OmpF is repressed; in media of low osmolarity, the reverse is true. It has been proposed that this shift in porin balance reflects an adaptation by *E. coli* and *S. typhimurium* to a transition from life in the animal gut to a free-living state or vice versa (37). Since the osmolarity of the intestinal contents is likely to be higher than that of the aqueous habitats of these bacteria, *ompC* expression will be favored in the gut and *ompF* expression will predominate when outside the host.

The porins span the outer membrane as protein trimers which admit small hydrophilic molecules to the cytoplasm. OmpF has been shown to form a significantly larger pore (diameter, 1.2 nm) than OmpC (diameter, 1.1 nm) (36). For this reason, growth conditions which alter the OmpF/OmpC ratio will affect the range of molecules admitted to the cell. Thus, the smaller pore size of OmpC (which predominates at high osmolarity) will aid in the exclusion of harmful molecules, such as bile salts, present in the gut. In the external aqueous environment, the larger pore size of OmpF will assist in scavenging for scarce nutrients.

In this study, a stable *ompR* mutation was introduced into a virulent strain of *S. typhimurium*. Since infection of an animal by the oral route will expose the bacterial cell to osmotic stress as described above, we were interested to discover whether loss of a key osmotic regulatory function compromised the ability of the cell to survive and grow in vivo. The effects of mutations in the structural genes *ompC*, *ompD*, and *ompF* were also investigated.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and growth conditions. The bacterial strains used in this study are listed in Table 1. Transductions were carried out using phage P22 *int-4* (46) as described previously (7). Vaccine strains were constructed by transducing transposon insertions into *S. typhimurium* SL1344. Following animal experiments, bacteria were recovered from the tissues and marker rescues were performed. For porin insertion mutations, this involved the analysis of the outer membrane protein patterns by polyacrylamide gel electrophoresis (see below) to ensure the absence of the relevant protein band. For the *ompR* mutation, the marker was rescued by transduction into a strain containing a *tppB*::Mu d1-8(*lac*) fusion and then this strain was tested for derepression of the fusion under anaerobic growth conditions (13). Bacteria were routinely cultured with aeration in liquid L medium (33) or on L agar plates unless otherwise specified. Ampicillin, kanamycin, and tetracycline were used at 50, 25, and 15 μg/ml, respectively. Genetically manipulated strains were routinely tested for serological characteristics with anti-H and anti-O diagnostic

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TABLE 1. Strains of *S. typhimurium* used in this study

Strain	Genotype	Source or reference
SL1344	*his*	B. A. D. Stocker (18)
CH1350	LT2 *opp250 ppB84*::Mu d1-8 *ompR1009*::Tn*10*	B. A. D. Stocker (18)
CJD359	SL1344 *ompR1009*::Tn*10*	This work
SH7241	LT2 *ompC396*::Tn*10*	This laboratory
BRD454	SL1344 *ompC396*::Tn*10*	This work
CH1420	LT2 *ompF*	This laboratory
BRD456	SL1344 *ompF1006*::Tn*10*	This work
CH338	LT2 *ompD156*::Tn*10*	This laboratory
BRD455	SL1344 *ompD159*::Tn*10*	This work

sera provided by Wellcome Diagnostics (Dartford, United Kingdom).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and cell envelope preparations. Polypeptide samples were prepared for gel electrophoresis and electrophoresed by the method of Laemmli (8, 21). Lipopolysaccharide structures were examined by using silver-stained polyacrylamide gel electrophoresis (50). Cell envelopes of *S. typhimurium* were prepared by using a procedure based on the method of Owen et al. (41). Overnight cultures of the strains in 250 ml of L broth were harvested by centrifugation and washed twice in phosphate-buffered saline (pH 7.2) and suspended in 20 ml of 0.05 M Tris hydrochloride (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride. The cell suspension was sonicated, broken debris was removed by centrifugation, and the cell envelopes were harvested by centrifuging at 100,000 × *g* for 2 h and suspended in 0.05 M Tris hydrochloride (pH 7.4).

Infection of mice and enumeration of bacteria in murine organs. Male BALB/c mice (8 to 10 weeks old) were used throughout. These were bred in the Animal Unit at Wellcome Research Laboratories. Livers, spleens, mesenteric lymph nodes, and Peyer's patches were homogenized as previously described (19, 27). Viable counts were performed on these homogenates as described previously (5, 40) with L agar as the growth medium. Counts with appropriate antibiotic supplements are shown in Fig. 2 as geometric means with standard errors (n = 4 mice per point). For oral inoculation of mice, bacteria were grown statically at 37°C overnight in 2 liters of L broth. The culture was pelleted by centrifugation and then suspended in 20 ml of phosphate-buffered saline. This was then further diluted in phosphate-buffered saline as required. Bacteria were administered orally in 0.2-ml volumes to lightly anesthetized mice by gavage needle. The bacterial count of the inoculum was calculated by plating appropriate dilutions on L agar plates. For intravenous (i.v.) inoculation, 0.2 ml of bacterial suspension was injected into the tail vein. Deaths were recorded over the following 4 weeks, and the 50% lethal dose (LD_{50}) was calculated by the method of Reed and Muench (42).

RESULTS

Characterization of *S. typhimurium omp* mutants in BALB/c mice. Defined mutations affecting outer membrane protein synthesis were transduced into the mouse virulent *S. typhimurium* SL1344 to assess the effects of these mutations on virulence. Transposon insertions were used to ensure complete inactivation of the required gene. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to assess individual transductants for smooth lipopolysaccharide biosynthesis and for altered production of outer membrane proteins caused by the introduced mutations. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis profiles of cell envelopes prepared from relevant strains are presented in Fig. 1. This shows the loss of the appropriate band for each outer membrane mutant. In this system OmpC and OmpF proteins run in the same region of the gel. Cell envelopes were prepared from cells grown in medium of high osmolarity, which favors expression of *ompC* and repression of *ompF*. Hence, for the *ompC* mutant (lane B) there are no bands in the higher-molecular-weight region as *ompF* is repressed. In lane D (*ompF* mutant) a band is still present because *ompC* is still expressed. Smooth isolates having the expected outer membrane protein profiles were selected and stored in glycerol at −70°C until required for challenge of mice. These strains are listed in Table 1. The mice were challenged orally to determine the LD_{50} for each strain. The $\log_{10}$ oral LD_{50} after 28 days was 6.38 for SL1344, >9.64 for CJD359, 6.71 for BRD454, 7.75 for BRD455, and 6.28 for BRD456. On repeated experiments, BRD456(*ompF*) and BRD454(*ompC*), administered orally, had $\log_{10}$ LD_{50} values similar to that for SL1344. BRD 455 (*ompD*) was still virulent after oral challenge, but the strain was consistently slightly less virulent than SL1344. However, CJD359 (*ompR*) showed attenuation after oral challenge, even at the highest dose administered. Thus, CJD359 is highly attenuated.

Because CJD359 was so highly attenuated, the i.v. LD_{50} was determined. Again, CJD359 was highly attenuated when compared with the parent strain, SL1344. CJD359 had an i.v. $\log_{10}$ LD_{50} of 5.13 compared with less than 1.0 for SL1344.

In vivo growth pattern of CJD359 after oral and i.v. administration to BALB/c mice. The ability of SL1344 and its *ompR* derivative CJD359 to grow in vivo after oral or i.v. administration was assessed. For the i.v. experiments, the numbers of viable organisms in the liver and spleen were ascertained at different days after challenge (Fig. 2A). After administration of 5×10^3 SL1344 cells, the bacteria grew rapidly in livers and spleens and all mice had died within 7 days of challenge. Following administration of 1.6×10^4 CJD359 cells, a level of about 10% of the inoculum was detected in the livers and spleens at 24 h after challenge. After this initial drop in bacterial cell numbers, CJD359 appeared to grow slowly and reach a maximal level of about 10^5 cells by day 14. Thereafter, the bacteria were slowly cleared. All mice challenged via the i.v. route with CJD359 exhibited a pronounced splenomegaly during the early phases of the infection which was similar to that detected previously with *aroA* derivatives of SL1344 (40).

BALB/c mice were inoculated orally with 6.3×10^9 SL1344 cells or 3.2×10^9 CJD359 cells. The numbers of bacteria in livers, spleens, Peyer's patches, and mesenteric lymph nodes were assessed at different times after challenge. Again, CJD359 exhibited an impaired ability to grow in vivo compared with SL1344 (Fig. 2B). SL1344 invaded the tissues

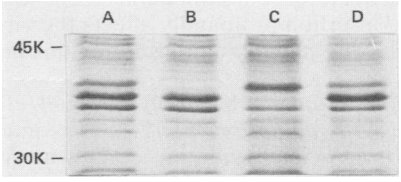

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell envelopes prepared from *S. typhimurium* SL1344 strains lacking different outer membrane proteins. Lanes: A, SL1344; B, SL1344 *ompC*; C, SL1344 *ompD*; D, SL1344 *ompF*. Molecular weights in thousands are indicated on the left.

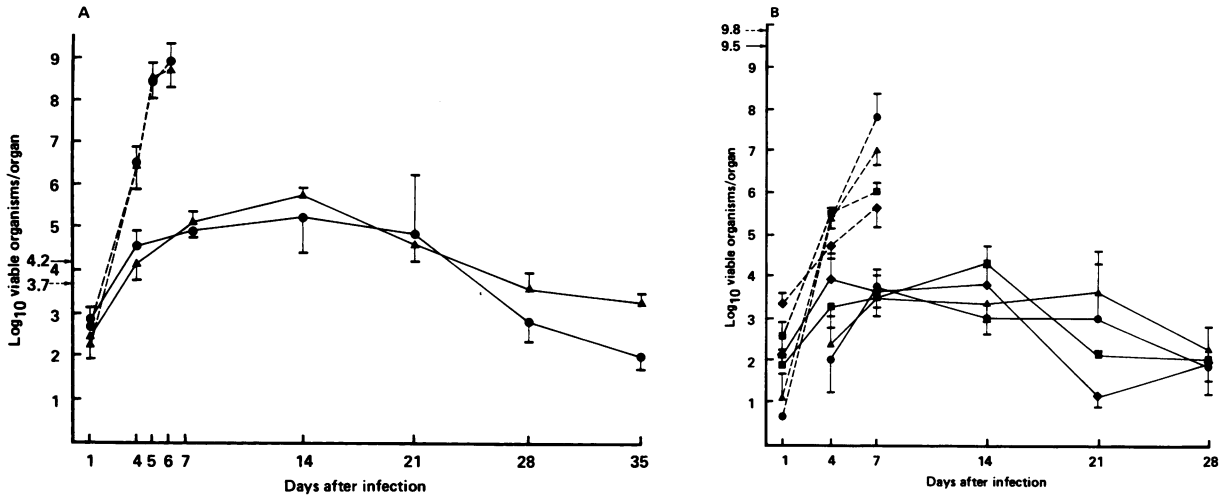

FIG. 2. Colonization of the tissues of BALB/c mice after i.v. (A) and oral (B) administration of SL1344 (- - - -) or CJD359 (———). ●, Livers; ▲, spleens; ■, mesenteric lymph nodes; ◆, Peyer's patches. The initial inocula are indicated by horizontal arrows: ---→, SL1344; →, CJD359. Each point represents the geometric mean plus or minus two standard errors for four mice.

of all mice challenged and grew rapidly, with all of the mice dying within 14 days of challenge. CJD359 also invaded the tissues, and bacteria were detected in Peyer's patches and mesenteric lymph nodes by day 1 postchallenge. By day 4, bacteria had reached liver and spleen, and they attained a maximal level of colonization of the host tissues by day 7. Thereafter, CJD359 was slowly cleared from the tissues.

Protection of mice after oral challenge. Mice were immunized orally with 10^{10} CJD359 cells and challenged orally 28 days later with the virulent parental strain SL1344. Mice vaccinated with CJD359 showed excellent protection against challenge with SL1344. The $\log_{10}$ LD_{50} in immunized animals was greater than 9.64, compared with 5.64 for unimmunized controls. Thus, mice vaccinated orally with CJD359 were well protected against virulent SL1344 challenge.

DISCUSSION

Although extensive studies have been made of the molecular properties of enterobacterial outer membrane porins, very little is known of their importance to the bacterium during in vivo growth. We have shown that mutations in some, but not all, porin-associated genes affect the virulence of *S. typhimurium* in a mouse model system. The *ompC*, *ompD*, and *ompF* genes encode pore-forming outer membrane proteins, whereas *ompR* is a porin regulatory gene. A mutation in the regulatory *ompR* locus is the most striking in its effects on virulence, with an *ompR* derivative of a virulent *S. typhimurium* strain being highly attenuated in vivo. The Tn*10* insertion in *ompR* may be polar on *envZ*, and the cell may be deficient for both functions (13). Certainly, the *ompR*::Tn*10* insertion profoundly affects the virulence of *S. typhimurium*. The underlying reason for this attenuation is not clear.

A mutation in *ompD* had only a small effect on the virulence of *S. typhimurium* SL1344. It is interesting that mouse deaths associated with this strain were spread over a number of doses (unpublished observations) in a manner analogous to that previously described for a *purE* mutant of *S. typhimurium* (40). The reasons for this effect are currently obscure; the *S. typhimurium* OmpD protein (3) is poorly characterized at the molecular level.

Mutations in *ompC* and *ompF* alone did not affect the virulence of *S. typhimurium* in the mouse system. Thus, strains mutated for either one of these functions are not impaired in their ability to grow in vivo. This finding is consistent with a previous report concerning a strain of *S. typhimurium* which had lost the ability to produce OmpC after treatment of an infected patient with cephalosporins (31).

It is possible that a strain harboring mutations in both *ompC* and *ompF* will be attenuated to the same degree as *ompR* mutants even though *ompR* mutants still express OmpD, and we are currently assessing this possibility. It is also unlikely that the loss of *tppB* expression (also *ompR* and *envZ* dependent) will impair virulence, as *S. typhimurium* also possesses a dipeptide (*dpp*) and an oligopeptide uptake (*opp*) system capable of substituting for the tripeptide transport function of *tppB* (16).

The OmpR and EnvZ proteins belong to a family of closely related regulatory elements known to respond to environmental stimuli (45). The key feature of this group is a partnership between an environmental sensor and a transcription activator. The group includes the NtrB-NtrC system of *E. coli*, which responds to nitrogen limitation (4, 17, 38, 43); the PhoR-PhoB system of *E. coli*, which responds to phosphate limitation (25, 26); and the CpxA-SfrA system of *E. coli*, which responds to dyes and other toxic compounds (2, 9, 47). An analogous system in *Rhizobium* spp. is DctB-DctD, which is responsive to 4C-dicarboxylic acids (44). In *Agrobacterium* spp., the VirA-VirG system responds to plant exudates (22, 49, 52). A system in a medically important pathogen which also appears to be related to OmpR-EnvZ is the ToxR-ToxS system of *Vibrio cholerae* (34). ToxR-ToxS is a positive regulator of the cholera toxin operon, *ctxAB*, and also regulates a number of other *V. cholerae* virulence determinants (32). The loss of *ompR-envZ* deprives the cell of part of its ability to interpret the external environment. The possibility exists that some specific genes, as yet unidentified, either on the *S. typhimurium* chromosome or on the virulence plasmid are *ompR-envZ* dependent.

Since little is known about the virulence factors of *Salmonella* species, programs to design live, attenuated oral vac-

cines for *Salmonella* species by rational means have concentrated on mutations in biosynthetic pathways. In general, the rationale behind this approach has been to produce avirulent derivatives of the pathogenic bacteria which are dependent on metabolites rarely found in mammalian tissues. Examples of attenuation by auxotrophy include *aro* (10, 18, 27, 40, 48) and *pur* mutants (30, 40). *Salmonella typhi* and *S. typhimurium* derivatives harboring these mutations individually, or as combinations, have been tested as vaccines in several animal species (40, 48). Another family of mutations that has enjoyed some success as vaccine strains includes *galE* lesions (23). A further class of attenuating lesions described previously includes mutations in the *crp* and *cya* genes of the *S. typhimurium* adenylate cyclase system (6). These mutations lead to reduced virulence and are effective as oral vaccines.

To our knowledge, the work described in this paper provides the first direct evidence that mutations in a gene coding for an outer membrane porin (OmpD) and an operon coding for a regulatory system involving an osmotic sensor and transcriptional activator of osmotically sensitive genes (OmpR-EnvZ) have an effect on the virulence of *Salmonella* species. We are carrying out further studies to elucidate the precise role of *ompR* in *Salmonella* virulence.

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