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Role of *ompR*-Dependent Genes in *Salmonella typhimurium* Virulence: Mutants Deficient in Both OmpC and OmpF Are Attenuated In Vivo

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A *Salmonella typhimurium* strain harboring stable mutations in both *ompC* and *ompF* was constructed from the mouse-virulent strain *S. typhimurium* SL1344. When administered orally to BALB/c mice the strain was attenuated, with the 50% lethal dose (LD_{50}) reduced by approximately 1,000-fold. However, the intravenous LD_{50} was reduced only by approximately 10-fold. The *ompC ompF* mutant persisted in murine tissues for several weeks following oral challenge, and mice immunized with this mutant were well protected against challenge with virulent SL1344. A strain harboring a stable mutation in *tppB* behaved in a manner similar to that of strain SL1344 in vivo, while a strain harboring mutations in *ompC*, *ompF*, and *tppB* behaved as an *ompC ompF* mutant in vivo, indicating that the *tppB* operon is not required for virulence in *S. typhimurium*.

Salmonella typhimurium is a facultative intracellular pathogen that naturally infects its mammalian host by the oral route. In susceptible mice, strains of *S. typhimurium* can cause a severe systemic disease resembling typhoid in humans. Entry into and passage through the alimentary tract expose bacteria to significant osmotic and other stresses. Part of the adaptive response of the bacteria to conditions of high osmolarity involves the preferential expression of one type of porin, OmpC, over another type, OmpF (18). Expression of the *ompC* and *ompF* genes is coordinately regulated at the level of transcription (7), involving an environmental sensor, EnvZ, an inner membrane protein of the histidine kinase class, and a cytoplasmically located transcriptional activator, OmpR. A recent study has shown that this two-component system is necessary for *Salmonella* virulence (4). A mutation in *ompR* rendered the organism avirulent in susceptible mice. However, in the same study, mutations in either *ompC* or *ompF* did not lead to attenuation of *Salmonella* virulence.

In this study, stable mutations in both *ompC* and *ompF* were introduced into a virulent *S. typhimurium* strain to assess the combined effects of these mutations on *Salmonella* virulence. The effects of mutations in *tppB* (encoding a tripeptide permease), another EnvZ/OmpR-dependent operon (5), were also tested.

The bacterial strains used in this study are listed in Table 1. Transductions were carried out by using bacteriophage P22 *int*4 (20) as previously described (3). Strains harboring mutations in *ompC*, *ompF*, and *tppB* were constructed by transducing transposon or Mu d insertions into *S. typhimurium* SL1344. Bacteria were routinely cultured with aeration in liquid L broth (14) or on L-agar plates unless otherwise specified. Ampicillin and tetracycline were used at concentrations of 50 and 20 μg/ml, respectively. Genetically manipulated strains were routinely tested for serological characteristics with anti-H and anti-O diagnostic sera provided by Wellcome Diagnostics (Dartford, United Kingdom).

Cell envelopes, protein, and lipopolysaccharide (LPS) samples were prepared for gel electrophoresis and separated on polyacrylamide gels by the method of Laemmli (11) as previously described (4).

Bacteria were grown as previously described (4) and used to infect innately salmonella-susceptible 8- to 10-week-old male BALB/c mice. The mice were bred in the animal unit at Wellcome Research Laboratories from breeders originally purchased from OLAC (Blackthorn, Bicester, Oxfordshire, United Kingdom). For in vivo persistence studies, livers, spleens, mesenteric lymph nodes, and Peyer's patches were homogenized and viable counts were determined as previously described (8, 13). Counts were expressed as geometric means ± 2 standard errors of the mean for four mice per point (see Fig. 1).

For determination of 50% lethal doses (LD_{50}s), groups of five mice were inoculated with serial dilutions of bacteria and deaths were recorded over the following 4 weeks. The LD_{50}s were calculated by the method of Reed and Muench (19).

Defined mutations affecting OmpC and OmpF synthesis were transduced into the mouse-virulent *S. typhimurium* SL1344 to assess the effects on virulence. Individual transductants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to ensure that they still synthesized complete LPS and were deficient in the porins OmpC and OmpF (data not shown). One mutant which fulfilled these criteria was designated CJD372 and was used for the construction of the *ompC ompF tppB* mutant and for the in vivo studies. The *tppB* phenotype of the *ompC ompF tppB* triple mutant was verified by rescuing the *tppB-lac* fusion into *S. typhimurium* LT2 by P22 transduction. This fusion was then tested for additive induction by anaerobiosis and leucine (10). The in vitro growth rate of CJD372 was also compared with that of the *ompR* mutant CJD359. The growth rate of CJD359 was three times greater than that of CJD372, with viable counts from an overnight statically grown culture of 4.8×10^8 and 1.5×10^8 CFU/ml, respectively.

These strains were characterized in vivo by using BALB/c

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

Strain	Genotype	Source or reference
SL1344	*his*	S. K. Hoiseth and B. A. D. Stocker (9)
CJD359	*ompR1009*::Tn*10*	Dorman et al. (5)
BRD454	SL1344 *ompC396*::Tn*10*	Dorman et al. (5)
CH1420	LT2 *ompF*1006::Mu d1-8 *ompR1009*::Tn*10*	Gibson et al. (6)
CJD372	SL1344 *ompC396*::Tn*10* *ompF1006*::Mu d1-8	This study
CJD408	SL1344 *tppB83*::Mu dJ	This laboratory
CJD409	SL1344 *ompC396*::Tn*10* *omp F1006*::Mu d1-8 *tppB* Mu dJ	This study

mice. The mice were challenged both orally and intravenously, and the LD_{50}s were determined on several occasions and the means were calculated. The results are shown in Table 2. The *ompR* mutant CJD359 was included as a control. This mutant was constructed as described previously (4). It can be seen that the *ompC ompF* mutant was highly attenuated orally, with the LD_{50} reduced by approximately $10^{2.6}$ compared with that of the wild-type SL1344. However, it was not as attenuated as the *ompR* mutant, despite the differences in the in vitro growth rates. It is also interesting to note that the deaths that occurred in mice infected with the *ompC ompF* mutant did not all occur at the highest dose but were spread over a number of doses (data not shown). The *tppB* mutant behaved exactly as strain SL1344, and the *tppB* mutation did not further attenuate the *ompC ompF* mutant when introduced into that strain. Results from the intravenous experiments demonstrated that for the *ompC ompF* mutant, the LD_{50} was reduced only by about 10-fold compared with that of the wild-type SL1344 and that deaths occurred in a dose-dependent manner. This is in contrast to results for the *ompR* mutant, for which the intravenous LD_{50} was reduced by approximately 10^5. Again, the introduction of the *tppB* mutation into the *ompC ompF* strain failed to attenuate the strain to the same degree as the *ompR* mutant.

The abilities of strain SL1344 and its *ompC ompF* derivative, CJD372, to persist in vivo were assessed after oral inoculation. BALB/c mice were inoculated with $\log_{10}$ 9.8 SL1344 cells or $\log_{10}$ 9.7 CJD372 cells, and the numbers of organisms in livers, spleens, Peyer's patches, and mesenteric lymph nodes were determined at different intervals after challenge. CJD372 exhibited an impaired ability to grow in vivo compared with that of strain SL1344 (Fig. 1). SL1344 invaded the tissues of all the mice challenged and grew rapidly, killing all of the mice within 7 days of challenge. CJD372 also invaded the tissues, with bacteria detected in Peyer's patches and mesenteric lymph nodes by day 1 postchallenge. It reached livers and spleens by day 4 and reached levels of $\log_{10}$ 5.5 cells in these organs by day 7. This level is 100-fold higher than that for *ompR* mutants in similar experiments (4). Thereafter, CJD372 was slowly cleared from the tissues. Again, there were some deaths of CJD372-infected mice over the duration of the experiment.

TABLE 2. Results of oral and intravenous infection of BALB/c mice with outer membrane mutants of virulent *S. typhimurium* strains

Inoculation route and strain	Log_{10} LD_{50} (cells)
Oral	
SL1344	6.7
CJD372 (*ompC ompF*)	9.2
CJD408 (*tppB*)	7.2
CJD409 (*ompC ompF tppB*)	8.8
CJD359 (*ompR*)	>9.92
Intravenous	
SL1344	<1.0
CJD372	2.2
CJD408	<1.0
CJD409	2.3
CJD359	5.18

Mice were immunized orally with $\log_{10}$ 9.47 CJD372 cells and challenged orally 70 days later with the virulent parent strain SL1344. Mice vaccinated with CJD372 showed excellent long-term protection against challenge, with a $\log_{10}$ LD_{50} of >10.2 versus 6.87 cells for the immunized mice. Again, there were deaths of mice infected with CJD372 prior to challenge (13 of 40). Thus, an *S. typhimurium* strain lacking both *ompC* and *ompF* porins is still able to protect mice after oral challenge.

This study has demonstrated that the insertional inactivation of both *ompC* and *ompF* results in the attenuation of strain SL1344. This finding is not unexpected, as it has previously been shown that *ompR* mutants are also attenuated (4). OmpR is a positive transcriptional regulator of OmpC and OmpF, and mutants deficient in this protein are generally unable to express these porins (21, 22). However, the *ompC ompF* mutants are not attenuated to the same level as *ompR* mutants, particularly when given by the intravenous route. The phenotypic effects of insertional inactivation of *ompR* would not necessarily be expected to be identical to those of inactivation of both *ompC* and *ompF*. This is because the porin genes are not the only genes under the control of *ompR*. Because the natural route of infection of *S. typhimurium* is oral, the organism is exposed to a variety of environmental stresses, including changes in osmolarity, pH, temperature, and oxygen availability. All of these environmental factors may play a role in altering the expression of the porin genes *ompC* and *ompF* (21, 22). The regulation of these genes is now known to be highly complex and involves transcriptional control not only via the histidine kinase sensor-transcriptional regulator EnvZ/OmpR system but also via histonelike proteins such as integration host factor (which affects levels of OmpF) (23). Other regulatory proteins that control levels of both OmpC and OmpF include OsmZ (6), DNA topoisomerase I (6), and the product of a poorly characterized locus, *envY*, which controls both *ompC* and *ompF* (12). There is also some posttranscriptional regulation of *ompF* via an untranslated antisense RNA species encoded by *micF* (17), whose expression is modulated by a minor outer membrane protein, TolC (16). Thus, although OmpR may be a central regulator of porin expression, it is not unique. These findings have recently been confirmed by a study that demonstrates that under appropriate environmental conditions, *ompC* and *ompF* can be induced in the absence of OmpR (2).

These factors may help to explain why the degree of attenuation achieved with an *ompR* mutant is not identical to that seen with an *ompC ompF* mutant. The *ompR* mutation is likely to be highly pleiotropic, whereas the mutations in the *ompC* and *ompF* structural genes are specific. The results of this study reveal the true contribution of the OmpC and OmpF porins to *S. typhimurium* virulence. While this contribution is highly significant, it is likely that there are

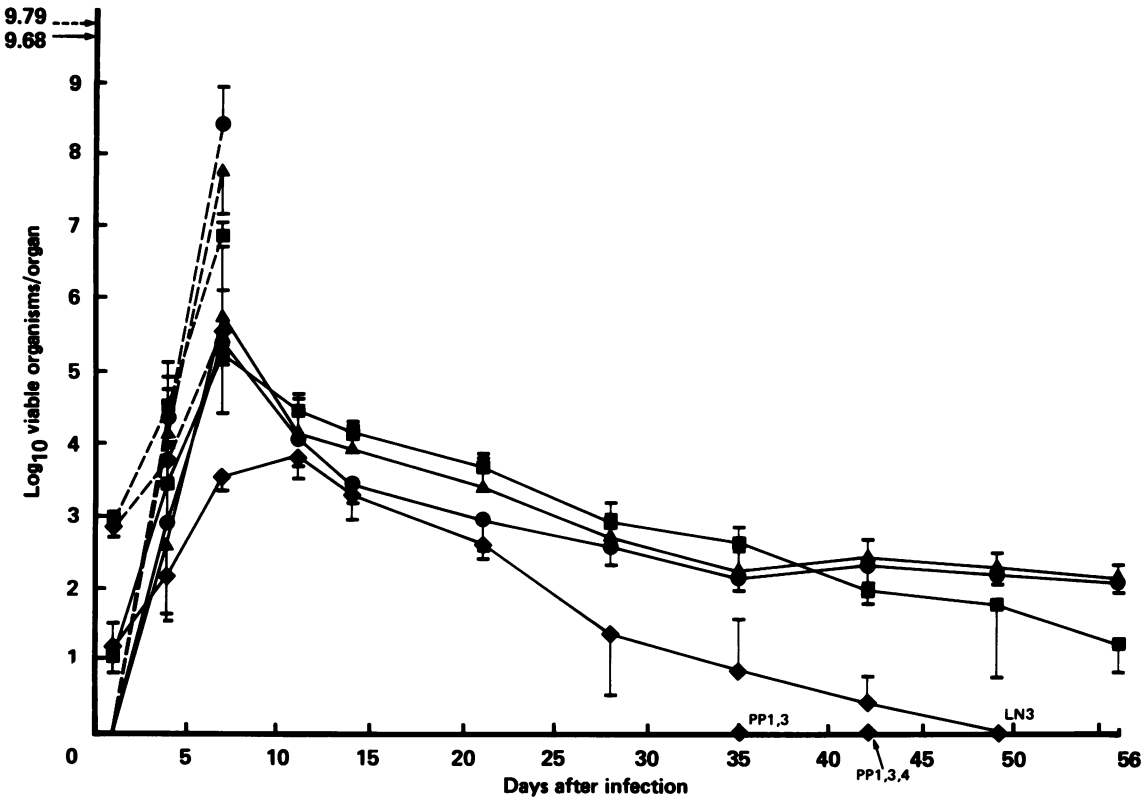

FIG. 1. Colonization of tissues of BALB/c mice after oral administration of SL1344 (———) or CJD372 (——). Symbols: ●, livers; ▲, spleens; ■, mesenteric lymph nodes; ◆, Peyer's patches. The initial inocula of SL1344 (--◗) and CJD372 (→) are indicated. Each point represents the geometric mean ±2 standard errors (bars) for four mice. Counts for individual mice when one or more of the group of four organs had no detectable salmonellae are indicated (e.g., PP1 and LN3).

other OmpR-regulated genes involved in virulence which have not yet been identified. We introduced a *tppB* mutation into the *ompC ompF* double porin mutant. The *tppB* operon is one of the few genetic elements identified as also being OmpR regulated. Experiments with the SL1344 *ompC ompF tppB* and SL1344 *tppB* derivatives demonstrated that this operon is not required for *S. typhimurium* virulence. This is not unexpected, since *S. typhimurium* possesses dipeptide and oligopeptide uptake systems capable of substituting for the function of *tppB* (5).

A further difference between mutations affecting regulatory and structural genes is that there exists the possibility of cross-talk between different members of the histidine kinase sensor-transcription activator family of gene regulators to which EnvZ/OmpR belongs (21, 22). Results from in vitro experiments have shown that sensors from one system can phosphorylate transcriptional activators from another (21, 22). In particular, it has been shown that OmpR can activate transcription of the *algD* gene from *Pseudomonas aeruginosa* in vivo (1). Thus, in an *ompR* mutant, *ompC* and *ompF* may be activated in an OmpR-independent manner, either through the action of other members of the sensor-regulator superfamily or by direct influence of the environment on the porin gene promoters, possibly via changes in DNA supercoiling (2, 6). Such activation is not possible in a strain containing insertional mutations in both *ompC* and *ompF*. This could be an explanation for the route-dependent nature of attenuation seen in *ompR* mutants. These mutants appear highly attenuated when given by the oral or intravenous route (4) but apparently not when given via the intraperitoneal route (15).

The finding in this study that an *ompC ompF* double mutant of *S. typhimurium* is attenuated orally but shows little loss of virulence when given intravenously may reflect the osmotic stresses imposed upon *S. typhimurium* when entering the host by the natural route. The results reported here help to clarify the nature of the *S. typhimurium* virulence network that is under the control of *ompR*. Clearly other factors involved have yet to be identified, and further experiments are in progress to elucidate them.

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