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Salmonella enterica Serovar Typhimurium *ompS1* and *ompS2* Mutants Are Attenuated for Virulence in Mice

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***Salmonella enterica* serovar Typhimurium mutants with mutations in the *ompS1* and *ompS2* genes, which code for quiescent porins, were nevertheless highly attenuated for virulence in a mouse model, indicating a role in pathogenesis. Similarly, a strain with a mutation in the gene coding for LeuO, a positive regulator of *ompS2*, was also attenuated.**

Salmonella enterica serovar Typhimurium pathogenesis is a complex multifactorial process that results from the activities of many bacterial gene products. It has been estimated that at least 4% of its genome is involved in virulence, and both in vitro and in vivo models have been used to better understand the infectious process (5, 17).

The *ompC* and *ompF* genes encode the two major porins of *Escherichia coli* and *S. enterica*. In *S. enterica* serovars Typhi and Typhimurium, the *ompS1* and *ompS2* genes code for two other porins that are expressed at very low levels, relative to the major porins OmpC and OmpF, under standard laboratory conditions. As for OmpC and OmpF, OmpS1 and OmpS2 are regulated by the EnvZ/OmpR two-component signal transduction system. In addition, *ompS1* is negatively regulated by the HN-S nucleoid protein, and *ompS2* is positively regulated by the LeuO regulator (10, 11, 26, 27).

Porins have been shown to trigger multiple synergistic signal transduction pathways in *Salmonella* infections, as they induce cellular activation, cytokine release, and the activation of protein tyrosine kinase, protein kinase A, and protein kinase C (14, 16). A role for the *S. enterica* serovar Typhimurium OmpC protein in initial recognition by macrophages and the distinction of regions of this protein that potentially participate in host cell recognition of bacteria by phagocytic cells have been documented (25). In addition, OmpS1 appears to have a role in the *Salmonella* life cycle, since mutations causing defects in swarming motility and biofilm formation have been found to map in *S. enterica* serovar Typhimurium *ompS1* (32, 34). Moreover, vaccination of mice with *S. enterica* serovar Typhi and Typhimurium porins confers protective immunity against live bacteria (18, 21, 24), and specific humoral and cellular immune responses are mounted against *S. enterica* serovar Typhi outer membrane proteins in typhoid fever patients, which has allowed the design of novel diagnostic assays (1, 6, 33).

It has been reported that porins play a role in virulence. For *Shigella flexneri*, both Δ*ompB* (Δ*ompR envZ*) and Δ*ompC* mutants were affected in two key steps for pathogenesis, i.e., in spreading from one epithelial cell to another and in host cell killing (2, 3). For *S. enterica* serovar Typhimurium, mutants lacking the *ompR* gene and a double *ompF ompC* mutant showed increased oral 50% lethal doses (LD_{50}), by >1,600- and 300-fold, respectively, relative to the wild type. Interestingly, a single *ompF* or single *ompC* mutant did not show attenuation (7, 9). Furthermore, *ompR* mutants were not cytotoxic to macrophages (23). Moreover, the EnvZ/OmpR two-component system has been shown to regulate *Salmonella* pathogenicity island 2 (15, 19, 22).

To define the role of the *ompS1* and *ompS2* genes in serovar Typhimurium regarding virulence, we constructed mutants (Table 1) of *Salmonella* in which the *ompS1* and *ompS2* genes were deleted by a one-step nonpolar gene inactivation procedure and replaced with selectable antibiotic resistance gene markers (8). The same procedure was used to obtain deletions of *ompR*, *envZ*, and *leuO* (Table 1). The antibiotic resistance gene was generated by PCR by using primers with 42-nucleotide homology extensions, and different primer sets were used to verify the presence of the antibiotic resistance gene cassette for kanamycin or chloramphenicol (Km^r and Cm^r, respectively) and of junctional sequences in the mutant, as described previously (8) (Table 2). The *ompS2* mutant was further characterized by the lack of induction of the OmpS2 porin in the presence of the cloned LeuO positive regulator (Fig. 1, lane 2). In contrast, the expression of LeuO from a cloned plasmid rendered OmpS2 expression in the wild-type control, as previously observed for *S. enterica* serovar Typhi (Fig. 1, lane 1) (10). The *ompS1* mutant was not verified in this manner, as there is no known positive regulator that causes its overexpression (11). Furthermore, both the *ompR* and *envZ* mutants lacked the presence of the OmpC and OmpF porins, which were restored upon complementation solely with the corresponding *ompR* and *envZ* cloned genes, respectively (Fig. 1, lanes 4 to 7). This observation particularly illustrated the nonpolar effects generated by the technique of Datsenko and Wanner (8), as the *ompR* and *envZ* genes are in close proximity, forming the *ompB* operon.

There were no observable differences between the wild-type strain and the isogenic *ompS1* and *ompS2* mutants of *S. en-*

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TABLE 1. *S. enterica* serovar Typhimurium strains and plasmids used for this study

Strain or plasmid	Genotype and/or relevant marker(s)	Reference or source
Strains		
ATCC 14028	Wild type (Sp^r)	American Type Culture Collection
STMS14	ATCC 14028 Δ*ompS1*::Km	This study
STMS23	ATCC 14028 Δ*ompS2*::Km	This study
STMR	ATCC 14028 Δ*ompR*::Km	This study
STMZ	ATCC 14028 Δ*envZ*::Cm	This study
STMLEUO1	ATCC 14028 Δ*leuO*::Km	This study
STMS23S15	STMS23 Δ*ompS1*::Cm	This study
STMD	ATCC 14028 Δ*ompD*::Km	This study
STMA1	ATCC 14028 Δ*invA*::Cm	This study
Plasmids		
pKD46	*oriR*101ts, λ Red recombinase system under p*araB* promoter control; Amp^r	8
pKD4	pANTSγ derivative containing an FRT-flanked Km^r gene from pCP15	8
pKD3	pANTSγ derivative containing an FRT-flanked Cm^r gene from pSC140	8
pFMtrc12	P15A1 *lacI trcp* Ap^r	10
pFMtrcleuO	Vector pFMtrc12-derived plasmid carrying the *leuO* gene	10
pFM2001	Vector pACYC184-derived plasmid carrying the *ompR* gene	This study
pROZ	Vector pMPMA6-derived plasmid carrying the *envZ* gene	This study

terica serovar Typhimurium in growth rates in LB or nutrient broth at a low or high osmolarity, in RPMI medium, or in Dulbecco's modified Eagle's medium (data not shown).

S. enterica serovar Typhimurium *ompS1* and *ompS2* mutants were compared with the wild-type strain, 14028, in a mouse model of infection. For inoculation, bacteria were grown in LB with shaking to an optical density at 540 nm of 0.6. Groups of six innately *Salmonella*-susceptible 6- to 8-week-old female

TABLE 2. Oligonucleotide primers used for this study

Primer	Sequence (5′-3′)
Primers for mutant construction[a]	
ompS1-H1P1	ATAATGAACAGAAAAGTTCTGGCACTGCTTGTCCCGGCGTTATTGTAGGCTGGAGCTGCTTCG
ompS1-H2P2	TTAGAACTGGTAGACCAGACCAACACCTACGATATCATCGGTCATATGAATATCCTCCTTAG
ompS2-H1P1	GATAAAAAAATGAAAAGAAAAGTATTGGCACTTGTCATCCTGTAGGCTGGAGCTGCTTCG
ompS2-H2P2	GAATAATTAGAACTGGTAAACCATACCCAGCGCGACGATATCCATATGAATATCCTCCTTAG
envZ-H1P1	GCCCGCACGCTGTTGCTCATCGTCACCTTGCTGTTCGTCATGTAGGCTGGAGCTGCTTCG
envZ-H2P2	TCCCCTGGACGCGAGCCACAGGAACCGGTAGCCAGGCGCGCATATGAATATCCTCCTTAG
ompR-H1P1	GAGAATTATAAGATTCTGGTGGTTGATGACGATATGCGTCTGTGTAGGCTGGAGCTGCTTCG
ompR-H2P2	GACGTAGCCCAGGCCCCATACGGTCTGAATATAACGCGGCATATGAATATCCTCCTTAG
leuO-H1P1	AAAACCGAAAAGCCGCATCTTTTAGATATGGGCAAACCACTGTAGGCTGGAGCTGCTTCG
leuO-H2P2	TATCGCTTACAAACAGAGACTAATAAATCTTCCATCCATTCATATGAATATCCTCCTTAG
ompD-H1P1	TTGAGGAAACACGCTAAGAAAATTATAAGGATTATTAAAATGTGTAGGCTGGAGCTGCTTCG
ompD-H2P2	CCCTGAAAGGACTGGCTTTGTATTCAGACTACAACAAAATTACATATGAATATCCTCCTTAG
invA-H1P1	CTATAGTGCTGCTTTCTCTACTTAACAGTGCTCGTTTACGACCTGTAGGCTGGAGCTGCTTCG
invA-H2P2	CCTTATATTGTTTTTATAACATTCACTGACTTGCTATCTGCTACATATGAATATCCTCCTTAG
Primers for mutant verification[b]	
k1	CAGTCATAGCCGAATAGCCT
k2	CGGTGCCCTGAATGAACTGC
kt	CGGCCACAGTCGATGAATCC
c1	TTATACGCAAGGCGACAAGG
c2	GATCTTCCGTCACAGGTAGG
ompS1-F	CATAATCAAAGCATCAAATAC
ompS1-R	CATCCATCATCTATAGTGGCA
ompS2-F	ACATAATAACAAATATATTCACCATAAATA
ompS2-R	TCTGTGCCCAGGGAACTGTGCGTTCTGTT
envZ-F	ATGAGGCGAATGCGCTTCTC
envZ-R	GCGCCGGACCGAAGCTGTAA
ompR-F	CATAACGTGATCGTCCCGACA
ompR-R	TAACTGCGTGCCGTCCTCCAG
leuO-F	CATTATGAATCGCAATGGTGTGAC
leuO-R	TTACGCCTTGATCGATAAGCGATC
ompD-F	CAGTCCCTATTTGAATTAATAC
ompD-R	CATAGACATCCAGCATTTCGA
invA-F	GATATTGCCTACAAGCATGAAATGG
invA-R	AAATGTTGCATAGATCTTTTCCTTA

[a] The nomenclature follows that used by Datsenko and Wanner (8) to indicate the forward (P1) and reverse (P2) primers with their homology extensions.
[b] The k and c primers correspond to the Km^r and Cm^r cassettes, as described previously (8); forward (F) and reverse (R) primers flank each gene.

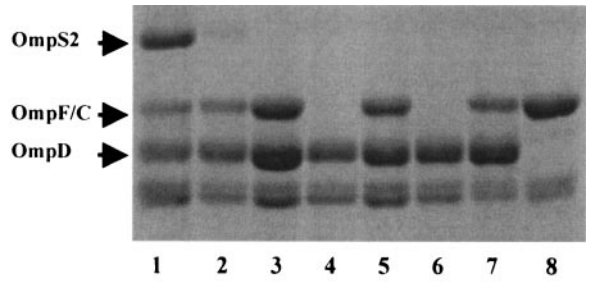

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel showing outer membrane protein profiles for various *S. enterica* serovar Typhimurium strains. Lanes: 1, STM14028 (wild type)/pFM*trcleuO* (plus 50 μM IPTG [isopropyl-β-D-thiogalactopyranoside]); 2, STMS23 (Δ*ompS2*::Km)/pFM*trcleuO* (plus 50 μM IPTG); 3, STM14028 (wild type)/pFM*trc*12 (plus 50 μM IPTG); 4, STMR (Δ*ompR*::Km); 5, STMR (Δ*ompR*::Km)/pFM2001 (*ompR*); 6, STMZ (Δ*envZ*::Cm); 7, STMZ (Δ*envZ*::Cm)/pROZ (*envZ*); and 8, STMD (Δ*ompD*::Km).

BALB/c mice (Harlan Sprague-Dawley Inc., Mexico) were inoculated orally with serial 10-fold dilutions of bacteria, and deaths were recorded over the following 28 days. Thirty minutes prior to oral infection, the gastric pH was neutralized by oral administration of 0.1 ml of 1% sodium bicarbonate. LD_{50}s were calculated by the method of Reed and Muench (28) in three to four repetitions. Mice inoculated with only phosphate-buffered saline (pH 7.4; PBS) were used as controls. *S. enterica* serovar Typhimurium strains STMS14 (Δ*ompS1*::Km), STMS23 (Δ*ompS2*::Km), and STMLEUO1 (Δ*leuO*::Km) were highly attenuated for oral infection (Fig. 2A).

To begin to understand the basis of the attenuation, mixed oral and mixed intraperitoneal infections (IPI) of mice were performed to establish a competitive index (CI) between the wild type and each particular mutant (4). Groups of six mice were inoculated either orally or intraperitoneally with 0.3 ml of an equal mixture of the strains, with total inocula of 10^8 and 10^5 bacteria, respectively. Mice were euthanized 6 and 3 days after oral and intraperitoneal infections, respectively, and spleens and livers were collected. Organs were homogenized in 5 ml of PBS. Resuspended cells were lysed by the addition of an equal volume of 0.5% sodium deoxycholate in PBS and incubated at room temperature for 15 min. Dilutions of cell lysates were plated on LB agar plates both with Km (40 μg/ml) or Cm (25 μg/ml) for enumeration of the CFU/mg of organ for the mutant strain and without antibiotics for enumeration of the mixture of the mutant and wild-type strains. CIs were calculated as described previously (4).

As shown in Table 3, the CIs in spleens and livers after oral infection for the *ompS1* and *ompS2* mutants indicated >250-fold reductions in survival, with 4- to 5-fold reductions in both spleens and livers after IPI. Furthermore, attenuation of the double Δ*ompS1*::Cm Δ*ompS2*::Km (STMS23S15) mutant was the predicted combined effect of the single mutations, suggesting that each mutation results in the loss of independent functions (Table 3). In addition, both the *ompS1* and *ompS2* mutants were impaired in the ability to cause bacteremia at an early time after infection (2 days), with CIs of $<1 \times 10^{-4}$. These results point towards a role of the *ompS1* and *ompS2* genes mainly in the initial stages of infection, since the virulence of these strains was not as severely compromised during IPI.

Bacterial shedding was determined by measuring the proportions of the wild type and the *ompS1*, *ompS2*, or *ompS1 ompS2* mutant in the feces after mixed oral inoculation as described above. Both the wild type and the mutants were shed in equal numbers at 1, 4, 8, 12, and 24 h postinfection: shedding was maximal at 4 h, at $3 \times 10^4 \pm 1 \times 10^4$ per mg of feces. The shed *Salmonella* cells were identified on MacConkey plates by the use of spectinomycin, and the mutants were identified by the use of spectinomycin and kanamycin. The identity of the shed *Salmonella* was further verified by streaking onto LB plates with the appropriate antibiotics. Groups of four mice were used for each mixed inoculation, and the experiment was done twice. In accordance with the shedding experiments, no particular sensitivity of the *ompS1*, *ompS2*, or double *ompS1*

A.

STRAIN	LD_{50} AFTER ORAL INFECTION
14028 (wild-type)	1.52×10^4
STMS14 (Δ*ompS1*::Km)	1.53×10^8
STMS23 (Δ*ompS2*::Km)	$>6.0 \times 10^8$
STMLEUO1 (Δ*leuO*::Km)	$>1.0 \times 10^9$

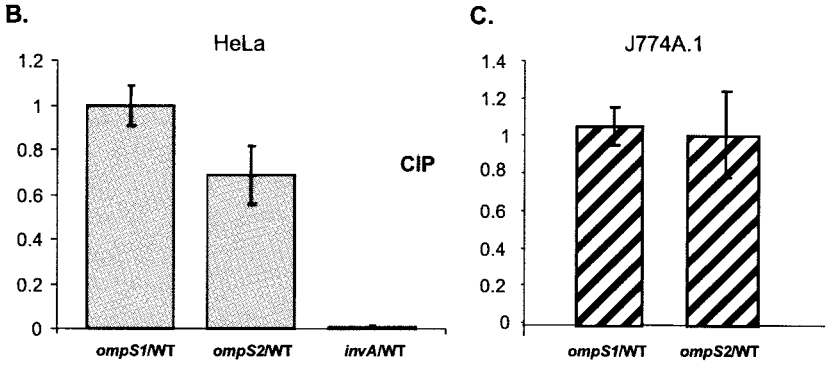

FIG. 2. Phenotypes of *S. enterica* serovar Typhimurium STMS14 (Δ*ompS1*::Km) and STMS23 (Δ*ompS2*::Km) mutant strains. (A) LD_{50}s of *S. enterica* serovar Typhimurium strains in BALB/c mice after oral infection. The values are the means of three to four independent experiments. The LD_{50} of the mutant with a deletion of the gene for LeuO, the positive regulator of *ompS2*, is also shown. (B) CII of mutants upon mixed infection with the wild-type strain in cultured HeLa cells. The CII was calculated by obtaining the ratio between the two strains in the output (intracellular bacteria recovered 3 h after infection) and dividing it by their ratio in the input (initial mixed inoculum). (C) CIP upon mixed infection of the J774.A1 macrophage line with either the STMS14 or the STMS23 mutant and the wild type. The CIP was calculated by obtaining the ratio between the two strains 24 h after infection and dividing it by their ratio 1 h 5 min after infection. For panels B and C, all values are means ± standard deviations of at least two experiments done in triplicate ($P < 0.001$ by Student's *t* test).

TABLE 3. Competitive indices of mixed oral and mixed intraperitoneal *S. enterica* serovar Typhimurium infections in BALB/c mice

Mutant mixed with wild type	Infection route[a]	CI of infection in indicated organ[b]	
		Spleen	Liver
ompS1	Oral	$3.8 \times 10^{-3} \pm 1.0 \times 10^{-3}$	$3.8 \times 10^{-3} \pm 1.2 \times 10^{-3}$
	i.p.	0.25 ± 0.11	0.28 ± 0.17
ompS2	Oral	$3.4 \times 10^{-3} \pm 9.0 \times 10^{-4}$	$2.6 \times 10^{-3} \pm 9.3 \times 10^{-4}$
	i.p.	0.19 ± 0.11	0.26 ± 0.12
envZ	Oral	$2.3 \times 10^{-5} \pm 9.0 \times 10^{-6}$	$2.6 \times 10^{-5} \pm 1.7 \times 10^{-5}$
	i.p.	$4.8 \times 10^{-4} \pm 1.4 \times 10^{-4}$	$6.8 \times 10^{-4} \pm 3.2 \times 10^{-4}$
ompR	Oral	$6.6 \times 10^{-6} \pm 5.7 \times 10^{-6}$	$8.7 \times 10^{-6} \pm 9.0 \times 10^{-6}$
	i.p.	$2.2 \times 10^{-4} \pm 4.2 \times 10^{-5}$	$4.4 \times 10^{-4} \pm 4.0 \times 10^{-4}$
leuO	Oral	$2.0 \times 10^{-3} \pm 1.3 \times 10^{-3}$	$1.0 \times 10^{-3} \pm 1.1 \times 10^{-3}$
	i.p.	0.12 ± 0.051	0.11 ± 0.041
ompS1 ompS2	Oral	$3.4 \times 10^{-5} \pm 1.6 \times 10^{-5}$	$2.5 \times 10^{-5} \pm 2.4 \times 10^{-5}$
	i.p.	$9.0 \times 10^{-3} \pm 3.0 \times 10^{-3}$	$1.3 \times 10^{-2} \pm 1.0 \times 10^{-2}$

[a] Organs from oral infections were collected at 6 days postinoculation; organs from IPI infections were collected at 3 days postinoculation.
[b] The CI was calculated as the ratio between the mutant strain and the wild type in the output divided by the ratio of the two strains in the input. All values are means ± standard deviations of at least two independent experiments. Differences between the wild type and each mutant were statistically significant ($P < 0.001$) by Student's *t* test.

ompS2 mutant was observed to a battery of stress factors compared to the wild type. The stress factors tested were as follows: lactic acid, 0.1 to 0.5%; pH, 3.5 to 6; bile salts, 3 to 7.5%; polymyxin B, 1 to 3 μg/ml; propionate, butyrate, and acetate, 50 to 200 mM; and deoxycholate or Triton X-100, 0.1 to 0.5%. Thus, attenuation of these mutants does not appear to be due to reduced survival in the gastrointestinal tract.

The CIs for the *envZ* and *ompR* mutants showed about 40,000- and 120,000-fold reduced abilities to colonize both the spleen and liver after oral infection, respectively, and about a 3,000-fold reduction after IPI (Table 3). Hence, the *ompR* mutant behaved as in previous studies, being highly attenuated in accordance with the proposed pleiotropic role for the OmpR regulator (7, 9, 19, 22, 23). Interestingly, the *envZ* mutant was also highly attenuated, indicating that it is the main phosphorylase for OmpR and that phosphorylation by other regulators or by acetyl-phosphate is not as relevant in vivo, in agreement with studies of the regulation of the *ssrAB* genes in *Salmonella* pathogenicity island 2 (19). Complementation in mice of the phenotypes of the *ompS1* and *ompS2* mutants was not feasible, although it was possible for bacteria grown in laboratory media (Fig. 1), since the vector plasmids were not stable in the host. Thus, future studies are aimed at generating stable constructs that will allow such complementation in the mice, although the technique of Datsenko and Wanner (8) has been designed to avoid polar effects.

Strains 14028 (wild type), STMS14 (Δ*ompS1*::Km), and STMS23 (Δ*ompS2*::Km) were used in mixed infections in vitro to determine the competitive indices of invasion (CII) of the human epithelial HeLa cell line (ATCC CCL-2) and in survival/proliferation (CIP) in the murine phagocytic cell line J774.A1 (ATCC TIB-67) (29). Invasion assays were done with a modified version of a previously described gentamicin protection assay (30), using 5×10^4 cells per well (90% confluent). Bacteria were grown under low-oxygen conditions and collected at the early/mid-logarithmic growth phase (optical density at 590 nm, ~0.2) as described previously (12, 20). A 1:1 mixture of the mutant and wild-type bacterial strains was applied at a multiplicity of infection of 100. For the survival/proliferation assay, a 1:1 mixture of two bacterial strains was prepared in PBS and added to the macrophages at a multiplicity of infection of 10 (30). As shown in Fig. 2B, no significant differences were observed between the mutants and the wild type regarding epithelial cell invasion compared with the *invA* mutant (STMA1 strain), which was severely impaired (13). Likewise, no difference was observed regarding survival/proliferation in mouse macrophages (Fig. 2C).

Thus, the attenuated phenotypes in the host were not evident upon assays in vitro. In this respect, the observations presented here are similar to those of other studies, where some *S. enterica* serovar Typhimurium mutants have been identified as being attenuated intraperitoneally and intragastrically yet show comparable invasion of cultured epithelial cells and survival in macrophages to those of the wild-type strain (5).

In agreement with the role of LeuO as a positive regulator of *ompS2* expression, it also appears to play a role in the initial stages of infection (Table 3). The CIs for the *leuO* mutant indicated 500- to 1,000-fold reduced survival in the spleen and liver, respectively, after oral infection and a <10-fold reduced survival after IPI in both the spleen and liver. However, it remains to be seen if LeuO regulates the expression of other genes required for the initial virulence events during *Salmonella* infection. This is in accordance with the recent identification of LeuO as a virulence factor in the *Caenorhabditis elegans* model of *Salmonella* infection (31).

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