Role of Periplasmic Peptidylprolyl Isomerases in *Salmonella enterica* Serovar Typhimurium Virulence

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FkpA is a peptidylprolyl isomerase whose expression is regulated by the alternative sigma factor, sigma factor E (σ^E). In contrast to the results of a previous report, inactivation of *fkpA* was found to have only a minor effect on the ability of *Salmonella enterica* serovar Typhimurium to invade and survive within epithelial and macrophage cell lines and cause infection in mice. However, an effect of the *fkpA* mutation on serovar Typhimurium virulence was seen if the mutation was combined with mutations in *surA* or *htrA*, two other σ^E-regulated genes, which encode proteins involved in protein folding and/or degradation in the periplasm.

Salmonella spp. possess a number of regulatory systems that allow them to sense and adapt to adverse conditions, primarily by altering the expressions of particular genes. One such system that is important for survival of *Salmonella enterica* serovar Typhimurium in vivo and in the environment is controlled by the alternative sigma factor RpoE (σ^E) (10). Two σ^E-regulated genes have been shown to be involved in *Salmonella* virulence, *htrA* (also known as *degP*) (10, 11) and *surA* (10, 14). Inactivation of *rpoE* has a far greater effect on serovar Typhimurium in vitro and in vivo than does inactivation of *htrA* or *surA* (10, 14). This indicates that other genes in the RpoE regulon may have a role in serovar Typhimurium virulence.

A promising candidate for another σ^E-regulated gene involved in *Salmonella* virulence is *fkpA*. FkpA is a periplasmic peptidylprolyl isomerase (PPIase). PPIases assist protein folding by catalyzing the normally slow *cis-trans* isomerization of prolyl residues in polypeptides (7). FkpA, like HtrA and SurA, has recently been shown to possess chaperone-like activity independent of its enzymatic activity (1, 2, 13). FkpA homologues are important for infection of host cells by other intracellular pathogens such as *Legionella pneumophila* and *Chlamydia trachomatis* (7, 9). In a previous study, an *fkpA* mutant of serovar Typhimurium biotype Copenhagen survived less well in macrophages and epithelial cells in vitro than did the parental wild-type (WT) strain (8). However, the role of *fkpA* in the virulence of serovar Typhimurium was not studied. Here we investigate the effect of inactivation of *fkpA*, alone or in combination with mutations in other σ^E-regulated genes, on the survival and invasion of serovar Typhimurium in different cell lines and in mice.

The complete serovar Typhimurium *fkpA* gene was isolated by PCR using primers designed from the *Escherichia coli fkpA* sequence. The predicted serovar Typhimurium FkpA protein exhibited 90% amino acid identity with its *E. coli* counterpart

(data not shown). This finding was confirmed with the publication of the complete genome sequence of serovar Typhimurium LT2 (12). The -35, -10, and intervening sequences of the *E. coli* σ^E-dependent promoter of *fkpA* were 100% conserved in the upstream region of the serovar Typhimurium *fkpA* gene (4) (data not shown). The *fkpA* gene was insertionally inactivated by introduction of a Km resistance cassette via a unique *Pst*I site within *fkpA* (nucleotide 306, amino acid 102). This construct was used to produce a serovar Typhimurium SL1344 *fkpA* mutant (GVB387) by allelic exchange using the suicide vector pRDH10 (10). The disruption of *fkpA* was confirmed by PCR and Southern hybridization (data not shown). We also constructed a serovar Typhimurium SL1344 *htrA fkpA* double mutant (GVB388) by P22 transduction of *fkpA*::Km^r into the serovar Typhimurium SL1344 Δ*htrA* strain BRD915 (6).

To examine whether FkpA is involved in the virulence of serovar Typhimurium, we determined whether the 50% lethal dose of GVB387 (*fkpA* mutant) following oral and parenteral (intravenous) infection differed significantly from that of the WT strain. There was no significant difference in the 50% lethal doses, or the times to death, of the WT and *fkpA* mutant strains by either route of infection (data not shown). To analyze further whether FkpA is involved in serovar Typhimurium virulence, the abilities of the WT and *fkpA* mutant strains to compete for growth in murine tissues were compared by competition assay (3). Three days after intraperitoneal inoculation, there were approximately twice as many CFU of the WT strain as of the *fkpA* mutant in the livers and spleens of mice, although this difference was not statistically significant (Table 1). However, the *htrA fkpA* double mutant exhibited a significant ($P < 0.05$) increase in attenuation over the single *htrA* mutant (competitive index [CI], 0.289) (Table 1).

Previously, it has been reported that FkpA was required for the survival of a serovar Typhimurium Copenhagen strain in macrophage (J774.A1) and epithelial (Caco-2) cell lines (8). We investigated whether our serovar Typhimurium *fkpA* mutant exhibited a defect in invasion or intracellular survival in the murine macrophage cell line RAW264.7 and the human

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TABLE 1. Competition assay with serovar Typhimurium strains[a]

Strains	CI
fkpA mutant versus SL1344	0.6[c]
fkpA htrA mutant versus *htrA* mutant	0.289[b]
fkpA mutant versus C5	1.1[c]
surA fkpA mutant versus C5	0.0121[b]
surA fkpA mutant versus *surA* mutant...........................	0.464[b]

[a] Mice were infected intraperitoneally with an inoculum containing ~10^3 CFU of both strains. Three days later, mice were killed and the number of CFU of each strain in the liver and spleen was determined. The CI was determined using the following formula: (input CFU of first strain/input CFU of second strain)/ (output CFU of first strain/output CFU of second strain). A Student's *t* test was used to determine whether the difference in the mean number of CFU recovered compared with the number of CFU inoculated for the pair of strains in each CI test was statistically significant. This was calculated by comparing the means of the ratio of the number of CFU recovered/the number of CFU administered for each strain.

[b] The difference in the recoveries of the two strains from murine tissue was significant ($P < 0.05$).

[c] The difference in the recoveries of the two strains from murine tissue was not significant.

epithelial cell lines Hep-2 and Caco-2. Both the *fkpA* and *htrA fkpA* mutants were found to invade RAW264.7 cells at a similar rate as SL1344 and BRD915 (*htrA* mutant), and thereafter all of the aforementioned strains grew intracellularly and there was no major difference in the numbers of bacteria isolated from the cells after 24 h (data not shown). A similar result was obtained with Hep-2 and Caco-2 cells (data not shown). The effect of inactivation of *fkpA* on the interaction of serovar Typhimurium with eukaryotic cells is contrary to that reported by Horne et al. (8). This may be due to the different genetic backgrounds of the bacterial strains used in the two studies. To ascertain whether this was the case, the *fkpA*::Km[r] mutation was transduced into a second WT serovar Typhimurium strain, C5, to generate strain GVB844. The survival of this mutant was analyzed in Caco-2 and RAW264.7 cells. In this case, there was a small, but significant ($P < 0.05$), effect of the *fkpA* mutation, as in both cell lines the number of GVB844 cells recovered after 24 h was lower than the number for C5 (Fig. 1).

E. coli possesses four genes encoding periplasmic PPIases, *fkpA*, *surA*, *ppiA*, and *ppiD*. In a search of the serovar Typhimurium genome database, a homologue for each of these genes was identified. It is possible that loss of FkpA may be compensated by the presence of other periplasmic PPIases. SurA has been shown to be involved in serovar Typhimurium pathogenesis, and recently its expression has been found to be regulated by σ^E in *E. coli* (5). We wished to examine whether a role for FkpA in serovar Typhimurium physiology in vivo and in vitro would be revealed in a strain that also lacked SurA. A *surA fkpA* double mutant was constructed by P22 transduction of the *fkpA*::Km[r] mutation from GVB387 into the C5 Δ*surA* strain BRD115 (14). The *surA* mutant invaded and survived less well than did C5 in both Caco-2 and RAW264.7 cells (24 h; $P < 0.05$) (Fig. 1), which is in agreement with the results of previous work (14). Inactivation of *fkpA* did not further reduce the ability of the *surA* mutant to invade or grow within eukaryotic cells (3 and 24 h; $P < 0.05$) (Fig. 1).

A competition assay was used to examine whether inactivation of *fkpA* decreased the ability of a *surA* mutant to grow and survive in murine tissues. The CI for the *surA fkpA* mutant versus the *surA* mutant was 0.464 ($P < 0.05$). This indicates
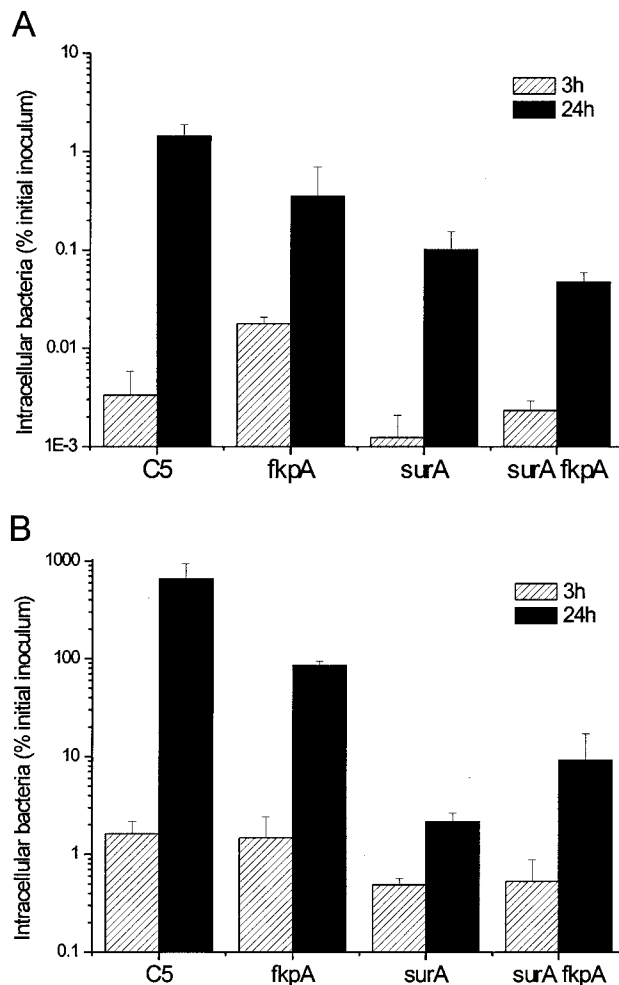

FIG. 1. Effect of mutations in *fkpA* and *surA* on the ability of serovar Typhimurium C5 to invade and replicate in epithelial and macrophage cell lines. The abilities of the different serovar Typhimurium strains to invade and grow in eukaryotic cells were examined using Caco-2 (A) and RAW264.7 (B) cells. Cells were infected with bacteria at a multiplicity of infection of ~1:1, and the assay was performed as described previously (10). The graphs indicate the viable bacteria recovered from inside the cells (as a percentage of the initial inoculum) at 3 and 24 h after infection. Each bar represents the mean number of CFU from triplicate experiments, and the error bars indicate standard deviations.

that lack of *fkpA* in a strain that also lacks *surA* does have a small effect on the ability of serovar Typhimurium to grow in vivo. The CI for the *surA fkpA* mutant versus C5 was 0.0121 (Table 1), indicating that the double mutant is highly attenuated ($P < 0.05$) compared with its parental WT strain. It was not possible to perform a competition experiment with the WT and *surA* mutant strains because the mutant lacks a selectable marker.

Contrary to the results of a previous report, this study indicates that FkpA is not crucial for normal survival or growth of serovar Typhimurium within phagocytic or nonphagocytic cells in vitro. FkpA is also dispensable for oral or parenteral infection of mice. A small effect on the in vivo growth of a serovar Typhimurium *fkpA* mutant was evident if the strain also lacked either SurA or HtrA, both of which are also involved in protein

folding in the periplasm of serovar Typhimurium. We are currently identifying other members of the σ^E regulon of serovar Typhimurium and investigating their importance for *Salmonella* pathogenesis.

REFERENCES

1. **Arie, J. P., N. Sassoon, and J. M. Betton.** 2001. Chaperone function of FkpA, a heat shock prolyl isomerase, in the periplasm of *Escherichia coli*. Mol. Microbiol. **39:**199–210.
2. **Behrens, S., R. Maier, H. de Cock, F. X. Schmid, and C. A. Gross.** 2001. The SurA periplasmic PPIase lacking its parvulin domains functions in vivo and has chaperone activity. EMBO J. **20:**285–294.
3. **Beuzon, C. R., and D. W. Holden.** 2001. Use of mixed infections with *Salmonella* strains to study virulence genes and their interactions in vivo. Microbes Infect. **3:**1345–1352.
4. **Danese, P. N., and T. J. Silhavy.** 1997. The sigma(E) and the Cpx signal transduction systems control the synthesis of periplasmic protein-folding enzymes in *Escherichia coli*. Genes Dev. **11:**1183–1193.
5. **Dartigalongue, C., D. Missiakas, and S. Raina.** 2001. Characterization of the *Escherichia coli* sigma E regulon. J. Biol. Chem. **276:**20866–20875.
6. **Everest, P., G. Frankel, J. Li, P. Lund, S. Chatfield, and G. Dougan.** 1995. Expression of LacZ from the *htrA*, *nirB* and *groE* promoters in a *Salmonella* vaccine strain: influence of growth in mammalian cells. FEMS Microbiol. Lett. **126:**97–101.
7. **Hacker, J., and G. Fischer.** 1993. Immunophilins: structure-function relationship and possible role in microbial pathogenicity. Mol. Microbiol. **10:**445–456.
8. **Horne, S. M., T. J. Kottom, L. K. Nolan, and K. D. Young.** 1997. Decreased intracellular survival of an *fkpA* mutant of *Salmonella typhimurium* Copenhagen. Infect. Immun. **65:**806–810.
9. **Horne, S. M., and K. D. Young.** 1995. *Escherichia coli* and other species of the *Enterobacteriaceae* encode a protein similar to the family of Mip-like FK506-binding proteins. Arch. Microbiol. **163:**357–365.
10. **Humphreys, S., A. Stevenson, A. Bacon, A. B. Weinhardt, and M. Roberts.** 1999. The alternative sigma factor, E, is critically important for the virulence of *Salmonella typhimurium*. Infect. Immun. **67:**1560–1568.
11. **Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. Ali, I. Miller, and C. Hormaeche.** 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. Mol. Microbiol. **5:**401–407.
12. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. Nature **413:**852–856.
13. **Spiess, C., A. Beil, and M. Ehrmann.** 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. Cell **97:**339–347.
14. **Sydenham, M., G. Douce, F. Bowe, S. Ahmed, S. Chatfield, and G. Dougan.** 2000. *Salmonella enterica* serovar Typhimurium *surA* mutants are attenuated and effective live oral vaccines. Infect. Immun. **68:**1109–1115.

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