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Decreased Intracellular Survival of an *fkpA* Mutant of *Salmonella typhimurium* Copenhagen

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The *fkpA* gene of *Salmonella typhimurium* encodes a protein similar to the macrophage infectivity potentiator (Mip) proteins of *Legionella pneumophila* and *Chlamydia trachomatis*. Because Mip proteins enhance the ability of these intracellular pathogens to survive within macrophages and epithelial cells, we tested whether the product of the *fkpA* gene would have the same effect on the intracellular growth of a virulent strain of *S. typhimurium*. By a series of P22 transductions, the *fkpA* gene of *S. typhimurium* Copenhagen was replaced with the inactive *fkpA1*::Ω-Cm gene from *Escherichia coli*, creating the mutant *S. typhimurium* KY32H1. The Copenhagen and KY32H1 strains were equally able to enter Caco-2 cells (an epithelial cell line) and J774.A1 cells (a macrophage-like cell line). However, compared to the parent, the *fkpA* mutant survived less well in both types of cells during the first 6 h after infection. The number of viable intracellular *S. typhimurium* Copenhagen bacteria remained constant 6 h after infection of Caco-2 cells, but the viability of *S. typhimurium* KY32H1 decreased significantly by 4 h postinfection. The *fkpA* mutant also exhibited a reduced ability to survive intracellularly in J774.A1 cells as little as 2 h postinfection. Complementation of the *fkpA* mutation by a plasmid-borne wild-type *fkpA* gene from *E. coli* restored the ability of *S. typhimurium* KY32H1 to grow normally in J774.A1 cells. Thus, expression of the *mip*-like *fkpA* gene confers on *S. typhimurium* Copenhagen properties analogous to those mediated by the Mip proteins in other intracellular pathogens, suggesting that this mechanism may play a role in the virulence and/or intracellular growth of numerous bacteria.

When an animal ingests a pathogenic strain of *Salmonella typhimurium*, an infection can occur which advances in three stages: the bacterium adheres to intestinal epithelial cells, attached bacteria enter the host cells, and internalized bacteria survive and replicate. Ingested bacteria may remain in the epithelium, or they may migrate through this layer of tissue, enter macrophages by phagocytosis, and be carried throughout the body. This process has been well reviewed (13, 18). The ability of *S. typhimurium* to enter and grow within epithelial cells and macrophages is important for infection (12, 18, 20, 24), and mutants unable to survive the intracellular environment are avirulent (12, 20, 24, 25).

Another intracellular pathogen, *Legionella pneumophila*, expresses a protein, Mip, that helps the bacterium survive within macrophages and free-living amoebae (5, 10). The Mip protein has extensive homology throughout its carboxyl terminus to the family of FK506 binding proteins, and it exhibits peptidyl-prolyl *cis-trans* isomerase activity (10, 16). A Mip-like protein in *Chlamydia trachomatis* is similar in structure and function (21, 27, 28). Until recently, *mip*-like genes had been observed only in bacteria that were primarily intracellular parasites, suggesting that these genes might be unique to such organisms (8, 27). However, the *fkpA* gene of *Escherichia coli* is related to the *mip* genes of *L. pneumophila* and *C. trachomatis* (22), and similar genes are present in several representatives of the *Enterobacteriaceae*, some of which are not intracellular pathogens (22).

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A functional *fkpA* gene is not required for survival of *E. coli* under laboratory conditions (22). It may be, though, that the *fkpA* gene product contributes to intracellular survival of some members of the *Enterobacteriaceae* in a manner similar to the role played by the Mip protein of *L. pneumophila*. We addressed this question by inactivating the *fkpA* gene in *S. typhimurium* Copenhagen. Such a mutant survived less well than its parent within epithelial or macrophage cell lines, suggesting that Mip-like proteins in diverse organisms may play important roles in intracellular survival.

Bacteria, plasmids, phage, and probes. The bacterial strains used in this work are listed in Table 1 and were routinely propagated in Luria-Bertani medium (30). The pathogenic strain *S. typhimurium* Copenhagen was originally isolated from a chicken with clinically apparent salmonellosis (34). Plasmid pSH125 is a pBluescript SK$^-$ (Stratagene, La Jolla, Calif.) plasmid that carries the wild-type *E. coli fkpA* gene plus 750 bp upstream and 550 bp downstream of *fkpA* (22). No other complete open reading frame is present, and a terminator sequence separates the cloned *fkpA* gene from the extreme 5′ end of a partial open reading frame downstream (22). Plasmid pPR1347 is a cosmid that carries the *rfb* gene cluster and the *rfc* gene of a *Salmonella* group B serovar (33); it was supplied to us by P. R. Reeves. Transfer of pPR1347 by λ phage transduction was performed as described previously (33). Phage P22 *HT105/1 int-201* was described by Schmieger (40) and Anderson and Roth (2) and was obtained from M. Schmid. Preparation of P22 phage, P22-mediated transductions, and purification of non-P22 lysogens on green plates were performed as described previously (29). P22 phage containing *E. coli*-modified DNA were produced by infecting *E. coli* JM109(pPR1347) with P22 derived from *S. typhimurium*. Chromosomal and plasmid preparations, bacterial transformations, restriction enzyme digestions, and agarose gel electrophoresis were per-

TABLE 1. Bacterial strains

Strain	Genotype	Source (reference)
E. coli strains		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*), F′*traD36 proAB lacI*^{q}ZΔM15	Salmonella Genetic Stock Center (33)
SM10	λ *cos* lysogen	Salmonella Genetic Stock Center (33)
SMH75	W1485 *thi supE fkpA1*::Ω-Cm F^-	S. Horne (22)
S. typhimurium strains		
TR5877	*hsdL6 hadSA29* (r_{LT}^- m_{LT}^+ r_s^- m_s^+) *metA22 metE551 ilv-452 trpB2 xyl-404 rpsL120* (Str^r) H1-b H2-e,n,x ($Fels2^-$) *nml*	T. Elliot (9)
SE8710	*hisG46 mutS121*::Tn*10*	M. Schmid
TRS121	TR5877 *mutS121*::Tn*10* (P22 transduction from SE8710)	This work
TRSA1	TRS121 *fkpA1*::Ω-Cm (P22 transduction from *E. coli* SMH75 pPR1347)	This work
Copenhagen	Natural isolate from a sick chicken	L. Nolan (34)
KY32H1	Copenhagen *fkpA1*::Ω-Cm (P22 transduction from TRSA1)	This work

formed according to established procedures (38). A 1-kb *Hin*cII DNA fragment carrying the entire *fkpA* gene was used as a template to create a ^{32}P-labeled probe, using the random primer method (11), and Southern blotting was performed as described previously (41). Ampicillin (50 μg/ml) and chloramphenicol (50 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) were added for selection of plasmids and strains, as appropriate. Other chemicals were also from Sigma.

Intracellular-survival assays. Caco-2 (ATCC HTB 37), a human colon adenocarcinoma cell line, and J774.A1 (ATCC TIB 67), a murine monocyte/macrophage-like cell line (36), were obtained from the American Type Culture Collection (Rockville, Md.). These cells are well-characterized hosts for studies of *S. typhimurium* invasion and replication (13, 14, 24). Caco-2 cells were maintained in 80% modified Eagle's medium (MEM) (Gibco BRL, Gaithersburg, Md.) supplemented with 20% fetal bovine serum (Gibco) and 1% amino acids (Bio-Whittaker, Walkersville, Md.) at 37°C in an atmosphere of 5% CO_2–95% air. J774.A1 cells were maintained in Dulbecco minimal essential medium (DMEM) (Gibco) plus 10% fetal bovine serum (3). Pathogenic and mutant strains of *S. typhimurium* Copenhagen were grown in Luria-Bertani medium (30) with shaking for 18 h at 37°C, so that the cultures were in stationary phase at the time of the experiments. Bacteria were collected by centrifugation for 5 min at 3,000 × *g* and were washed three times with 1 ml of phosphate-buffered saline (PBS). The final suspension was diluted to 10^7 bacteria per ml.

To measure intracellular survival of *S. typhimurium* strains in Caco-2 cells, 1.5×10^5 Caco-2 cells were seeded onto Falcon multiwell tissue culture plates (Becton Dickinson Labware, Lincoln Park, N.J.) supplemented with 2 ml of medium (14). At this density, cells achieved confluence in about 6 days. Medium was replaced every 2 to 3 days, and cells were used for experiments between 7 and 10 days after plating. Confluent monolayers were rinsed once with 3 ml of prewarmed MEM and incubated with 2 ml of prewarmed MEM for 30 min at 37°C. This medium was discarded to remove serum components. The bacterial inoculum (1 ml per well) was added and the plates were centrifuged at 600 × *g* for 5 min to synchronize infection of the tissue culture cells (26). Infected cells were incubated at 37°C for 30 min in an atmosphere of 5% CO_2–95% air, rinsed five times with 3 ml of PBS to remove extracellular bacteria, and incubated for 90 min with MEM containing gentamicin (125 μg/ml) to kill extracellular adherent bacteria (the wild-type and mutant bacteria were equally sensitive to gentamicin). Afterwards, the monolayers were rinsed five times with 3 ml of PBS to remove residual antibiotic. Intracellular bacteria were released from sets of three wells at 30 min, 2 h, 4 h, or 6 h after infection by adding 1 ml of PBS containing 1% Triton X-100 and incubating at room temperature until the cells in the monolayer ruptured (~5 min). The bacteria were resuspended in this solution, diluted in PBS, and plated onto nutrient agar plates (30). These plates were incubated overnight, and colonies were counted to determine viable counts (26). The experiment was performed twice, viable counts for each time point were derived by averaging counts from each set of three wells.

To measure intracellular survival of *S. typhimurium* strains in J774.A1 cells, we used the in vitro assay described by Kusters et al. (23). J774.A1 cells were seeded at a density of approximately 5×10^5 cells per well in multiwell tissue culture plates, incubated for 18 h, and washed with DMEM prior to the assay. One milliliter of bacterial suspension (10^7 bacteria) in DMEM was added to each well of J774.A1 cells, and the plates were centrifuged at 600 × *g* for 5 min. Subsequent steps were identical to those described for the infection of Caco-2 cells.

Construction of an *fkpA* mutant of *S. typhimurium* Copenhagen. *S. typhimurium* Copenhagen is a pathogenic strain isolated from chickens with salmonellosis and is capable of intracellular growth. To test the effect of *fkpA* on intracellular survival of *S. typhimurium* Copenhagen, we replaced the *fkpA* homolog in this strain with the inactive *fkpA1*::Ω-Cm gene from *E. coli* SMH75. The replacement procedure was complicated by the fact that the DNA sequences of the two organisms differ by an average of ~15% (which inhibits homologous recombination) and by the fact that they have different restriction-modification systems (which reduces the efficiency of productive DNA transfer). These problems were circumvented by transferring *E. coli*-derived DNA to a strain of *S. typhimurium* in which the mismatch repair and restriction systems had been inactivated and then transferring the *Salmonella*-modified genes into *S. typhimurium* Copenhagen.

E. coli-modified phage P22 was obtained by infecting *E. coli* JM109(pPR1347) with a phage lysate from *S. typhimurium* (33). The *fkpA1*::Ω-Cm mutant of *E. coli* was made sensitive to phage P22 infection by introducing plasmid pPR1347 from *E. coli* SM10(pPR1347) into *E. coli* SMH75 by λ phage transduction (33). *E. coli* SMH75(pPR1347) was then infected with *E. coli*-modified P22, and the resulting lysate served as the source of virus for transduction of *fkpA1*::Ω-Cm into *S. typhimurium.*

E. coli genes fail to recombine into the chromosome of *S. typhimurium* because nucleotide differences in the resulting heteroduplexes are removed by the mismatch repair system (37). Therefore, we transduced a *mutS121*::Tn*10* mutation

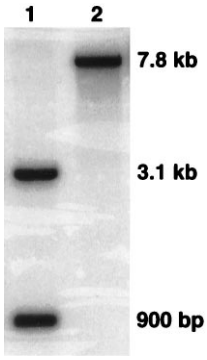

FIG. 1. Confirmation of *fkpA* insertion mutagenesis by Southern blotting. Chromosomal DNA from *S. typhimurium* Copenhagen (lane 1) and *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm) (lane 2) was isolated, digested with *Pst*I, separated by electrophoresis through a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to a DNA probe specific for the *fkpA* gene. *S. typhimurium* Copenhagen carries an *fkpA* homolog that has an internal *Pst*I site. Thus, when the *Salmonella* gene is present, the *fkpA*-specific probe hybridizes to two *Pst*I fragments (4 kb in combined length) (lane 1). The *fkpA* gene of *E. coli* is located on a single 4-kb *Pst*I DNA fragment. Replacement of the *S. typhimurium* Copenhagen *fkpA* homolog with the *fkpA1*::Ω-Cm allele from *E. coli* increases the size of the single 4-kb *fkpA*-specific *Pst*I fragment by ~3.8 kb (lane 2).

from *S. typhimurium* SE8710 into *S. typhimurium* TR5877 (creating strain TRS121) to inactivate this barrier. The restriction system of strain TR5877 is also inactive, which allowed us to transduce the *fkpA1*::Ω-Cm mutation from *E. coli* SMH75 (pPR1347) to *S. typhimurium* TRS121, creating strain TRSA1. *fkpA1*::Ω-Cm was moved from this strain by P22 transduction into wild-type *S. typhimurium* Copenhagen, creating *S. typhimurium* KY32H1.

The wild-type and mutant *fkpA* genes of *E. coli* and *S. typhimurium* can be distinguished by their restriction patterns (22). The presence of *fkpA1*::Ω-Cm and the absence of the wild-type gene in *S. typhimurium* KY32H1 were confirmed by Southern blotting (Fig. 1). The *fkpA* mutant of *S. typhimurium* Copenhagen grew normally, indicating that, as in *E. coli*, the gene is not required for growth under normal laboratory conditions (data not shown).

Mutation of *fkpA* decreases intracellular survival in epithelial cells. The wild-type and *fkpA* mutant strains of *S. typhimurium* Copenhagen were tested for their ability to survive intracellularly after infection of a monolayer of Caco-2 cells, an epithelial cell line. The wild-type strain and its *fkpA1*::Ω-Cm derivative entered Caco-2 cells equally well; 30 min after infection the numbers of intracellular bacteria of the two strains were approximately equal (Fig. 2). Thus, inactivation of *fkpA* did not affect entry of bacteria into epithelial cells. In addition, these strains persisted equally well within Caco-2 cells immediately after entry, in that they exhibited similar intracellular survival 2 h after infection. However, 4 h after infection, ~80% of intracellular *S. typhimurium* KY32H1 bacteria had been killed, and the number of bacteria did not recover by 6 h (Fig. 2). In contrast, the intracellular number of wild-type *S. typhimurium* Copenhagen bacteria remained relatively constant from 2 to 6 h (Fig. 2). Thus, inactivation of *fkpA* decreased the ability of *S. typhimurium* Copenhagen to survive intracellularly in Caco-2 cells.

Mutation of *fkpA* decreases intracellular survival in macrophages. The macrophage-like cell line J774.A1 (36) has been used extensively to test the susceptibility of intracellular pathogens to phagocytosis (3, 17). Some virulence loci of *S. typhimurium* affect survival in either epithelial cells or J774.A1 but not in both (17), raising the possibility that the *fkpA* mutant

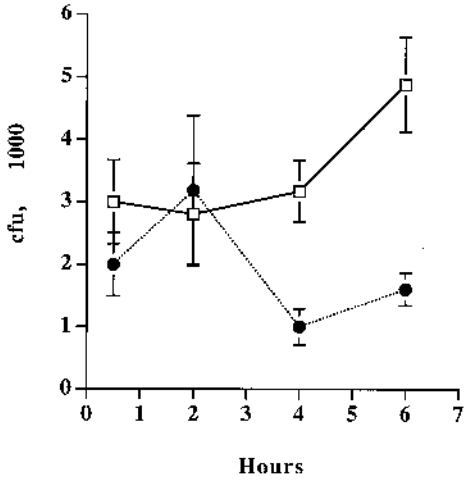

FIG. 2. Intracellular survival in Caco-2 cells of *S. typhimurium* Copenhagen and its *fkpA* derivative. *S. typhimurium* Copenhagen (open squares) and *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm) (closed circles) were added to cultured Caco-2 cells, and intracellular bacteria were released at various times after infection. At each time point, the number of viable CFU is the average of six separate Caco-2 cultures from two independent experiments. Error bars represent standard errors of the means. A one-factor analysis of variance and F test revealed that the differences between *S. typhimurium* Copenhagen and *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm) were significant at 4 and 6 h (*P* values of 0.0035 and 0.0041, respectively).

might behave differently toward the two types of cells. Therefore, we tested *S. typhimurium* Copenhagen and the *fkpA* mutant for intracellular survival after phagocytosis by J774.A1. As in the experiments with Caco-2 cells, the wild-type and mutant strains entered the J774.A1 cell line equally well (the numbers of viable intracellular bacteria were the same 30 min after

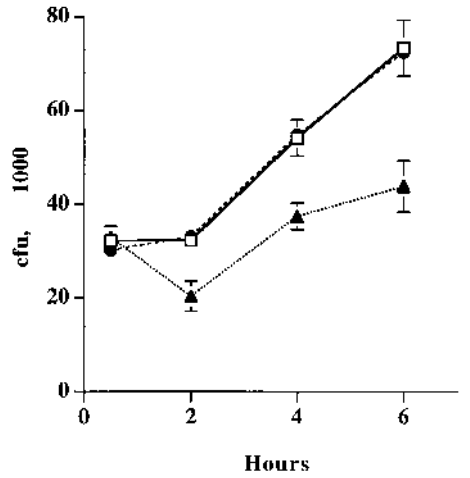

FIG. 3. Intracellular survival in J774.A1 cells of *S. typhimurium* Copenhagen and its *fkpA* derivative. *S. typhimurium* Copenhagen and *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm) were added to cultured J774.A1 cells, and intracellular bacteria were released at various times after infection. At each time point, the number of viable CFU is the average of six separate J774.A1 cultures from two independent experiments. Error bars represent standard errors of the means. A one-factor analysis of variance and F test revealed that the differences between *S. typhimurium* Copenhagen and *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm) were significant at 2, 4, and 6 h (*P* values of 0.0027, 0.0098, and 0.0035, respectively). Open squares, *S. typhimurium* Copenhagen; closed triangles, *S. typhimurium* KY32H1 (*fkpA1*::Ω-Cm); closed circles, *S. typhimurium* KY32H1(pSH125) (plasmid carrying the cloned wild-type *fkpA* gene from *E. coli*).

phagocytosis) (Fig. 3). However, at 2 h after infection the number of viable *fkpA* mutant cells was reduced (Fig. 3), indicating that a functional *fkpA* gene contributed to intracellular survival during the initial phase of infection of these phagocytes. After 2 h, differences between the numbers of viable intracellular wild-type and mutant bacteria persisted or increased (Fig. 3).

Because the *fkpA1*::Ω-Cm mutation was moved from *E. coli* to *S. typhimurium* Copenhagen by several steps of phage P22 transduction, it was theoretically possible that differences in intracellular survival might be due to transfer of a closely linked but unknown genetic difference other than *fkpA* inactivation. Therefore, we transformed *S. typhimurium* KY32H1 with plasmid pSH125, in which the only intact cloned gene is the wild-type *fkpA* from *E. coli* (22). The growth in J774.A1 cells of the mutant carrying the wild-type *fkpA* gene was identical to that of the wild-type parental strain (Fig. 3). Thus, the intracellular growth defect of *S. typhimurium* KY32H1 was complemented by a functional *fkpA* gene, indicating that the defect in *fkpA* was responsible for the original decrease in intracellular survival.

Similarities in the phenotypes of *fkpA* and *mip* mutants. The presence of an active *fkpA* gene enhanced intracellular survival of *S. typhimurium* Copenhagen in Caco-2 cells and in the J774.A1 macrophage cell line, especially during a relatively short period in the first few hours of infection. Wild-type *S. typhimurium* becomes resistant to intracellular killing by macrophages between 4 and 8 h postinfection (1), which is consistent with the time at which the *fkpA* mutant exhibited sensitivity to killing within epithelial cells and macrophages, suggesting a possible correlation. The behavior of the *fkpA* mutant appeared to be qualitatively similar to the survival of *L. pneumophila mip* mutants after phagocytosis by human alveolar macrophages (6) or after infection of protozoan hosts (7). In the case of *L. pneumophila*, the intracellular viability of *mip* mutants decreases over the first 24 h of infection (6, 7). A similar decrease in viable intracellular bacteria was observed during the initial 6 h after infection of epithelial cells or macrophages by an *fkpA* mutant of *S. typhimurium* Copenhagen. These results suggest that Mip-like proteins could play similar roles in the infection cycle of different bacteria.

The *mip* gene of *C. trachomatis* also contributes to the infectivity of that bacterium, although it is not known what stage is affected (27). In addition, Moro et al. have discovered that a *mip*-like gene in the intracellular protozoan parasite, *Trypanosoma cruzi*, contributes to its ability to infect epithelial cells (32). The parallels displayed by all these infections led Moro et al. to speculate that Mip proteins might help determine the wide host range of these intracellular pathogens (32). If this is true, then *S. typhimurium* would be a natural addition to this group because it, too, can infect a variety of host cells. However, the existence of very similar genes in at least 12 enterobacterial species (22) argues against the idea that possession of a *mip*-like gene is solely responsible for determining whether a bacterium is an intracellular pathogen. In fact, because the *fkpA* gene is present in so many bacteria, FkpA and other Mip-like proteins may have a function that is normally unrelated to infectivity.

A second *mip*-like (and *fkpA*-like) gene was discovered during sequencing of the *E. coli* genome (4). The predicted protein product of this gene (which we have named *fklB*) is highly similar to the Mip proteins of *L. pneumophila* and *C. trachomatis* and to the amino acid sequence of the product of the *E. coli fkpA* gene (35). Purified FklB protein binds FK506 and exhibits peptidyl-prolyl *cis-trans* isomerase activity, and, like *fkpA*, the *fklB* gene is present in various members of the *Enterobacteriaceae*, including *S. typhimurium* (35). These extensive similarities suggest that *fklB* may also contribute to intracellular survival of *S. typhimurium* Copenhagen and that the gene may partially substitute for the loss of *fkpA*, a possibility we are beginning to investigate.

How might the FkpA protein contribute to virulence of *S. typhimurium?* The family of FK506 binding proteins, of which FkpA is a member, are peptidyl-prolyl *cis-trans* isomerases that may be involved in protein folding (15, 39). In fact, recent evidence suggests that FkpA plays an active role in the correct folding of some periplasmic proteins in *E. coli* (31). Such an enzyme could play an indirect role in pathogenesis by determining the proper conformation of one or more bacterial proteins that are themselves virulence factors. Alternatively, FkpA or one of the proteins it acts upon might enhance the transition from extracellular to intracellular growth of bacteria. Finally, FkpA might promote virulence directly if its substrate is one or more defensive proteins of the host cell, or an unrecognized enzymatic capability might account for the contribution of FkpA to virulence. The fact that the *fkpA* gene appears in pathogenic and nonpathogenic bacteria does not diminish its importance, because many bona fide virulence factors of *S. typhimurium* also appear in nonpathogenic *E. coli* (19). It will be interesting to learn how FkpA fits into the complex web of events that constitute the virulence potential of the salmonellae and other organisms.

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