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Characterization of a Novel Porin Involved in Systemic 
Yersinia enterocolitica Infection

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Yersinia enterocolitica is an enteric pathogen capable of causing systemic disease in a murine model. We have identified a novel protein, systemic factor protein A (SfpA), conserved in other pathogenic bacteria that is involved in systemic disease. Analysis of bacterial colonization revealed that a ΔsfpA strain is defective in mesenteric lymph node colonization. Bioinformatics and functional studies suggest that SfpA is a porin.

The genus Yersinia includes three pathogenic species, Yersinia enterocolitica, Yersinia pseudotuberculosis, and Yersinia pestis, which share a common tropism for lymphoid tissue. Upon ingestion, Y. enterocolitica survives the harsh acidic environment of the stomach and progresses to the terminal ileum. Once there, the bacterium invades the specialized lymphoid tissue of the small intestine known as Peyer’s patches through interactions with intestinal M cells (5, 9, 12). Successful colonization of the Peyer’s patches can subsequently lead to dissemination to deeper tissues, including the mesenteric lymph nodes (MLN), spleen, and liver. Progression of infection to deeper tissues and pathology of these tissues are most commonly seen in immunocompromised individuals (3, 19).

Several factors that have been shown to be involved in various stages of Y. enterocolitica infection have been identified. During early stages of colonization, the outer membrane proteins Inv and YadA allow bacteria to adhere to, colonize, and invade the intestinal epithelium (13, 14, 22–24, 35). The Yops encoded by the virulence plasmid are essential at later stages of yersiniosis, presumably to counteract the immune response. Effectors of this system are injected into host cells to prevent phagocytosis by macrophages and hinder the release of proinflammatory cytokines from macrophages and epithelial and endothelial cells (31).

Previously, an in vivo expression technology screen was conducted to identify genes in Y. enterocolitica that are expressed during systemic infection (8). One gene identified in this screen, sif15, is not found in either Y. pseudotuberculosis or Y. pestis, and a strain carrying a mutation in sif15 was attenuated during intraperitoneal competitive infections with wild-type Y. enterocolitica. Furthermore, in vitro analysis demonstrated that sif15 transcription is significantly increased at 37°C (8). Based on analysis of the sequence around sif15 (YE3039), it does not appear to be part of an operon (http://www.sanger.ac.uk/Projects/Y_enterocolitica/). Subsequently, we renamed sif-15 with the designation sfpA (systemic factor protein A) in order to avoid confusion with Salmonella induced filaments (Sifs). In this study, we show that SfpA is located in the membrane and is necessary for sustained colonization of mesenteric lymph nodes. Furthermore, in vitro data suggest that SfpA forms a pore in the outer membrane when expressed in Escherichia coli.

Requirement for SfpA in mesenteric lymph nodes. To further define the role of sfpA in virulence, an in-frame deletion of sfpA was made in Y. enterocolitica strain JB580v (16). Flanking 500-bp regions of sfpA were cloned into the suicide plasmid pSR47S and subsequently used to exchange the sfpA gene with a kanamycin cassette by allelic exchange, as described previously (21). The absence of sfpA was confirmed by Southern blotting and PCR (data not shown), and the ΔsfpA mutant was called strain YVM1051. Complementation of the ΔsfpA mutation was carried out as follows. To construct the complementing sfpA strain, the pSR47S plasmid was engineered with regions carrying the intragenic sequence found between the putA gene (YE1933) and a phage tail gene (YE1931) flanking a multiple cloning site, and ΔsfpA was cloned into pPutA at the multiple cloning site, and sfpA was recombined by allelic exchange into the sfpA mutant strain (YVM1051) at the site between YE1933 and YE1931. The construct was confirmed by Southern blotting and PCR (data not shown). The complementing strain was called YVM1220.

To assess the effect of the ΔsfpA mutation on virulence, C57BL/6j mice were orally infected with approximately 1 × 10⁶ ΔsfpA bacteria from an overnight culture grown in Luria-Bertani broth at 26°C and compared to mice receiving an analogous inoculation with wild-type Y. enterocolitica (JB580v) (16). Infections were done as previously described (4, 11). Subsequently, Peyer’s patches, MLN, and spleens were harvested at 1, 3, and 7 days postinfection, and the number of CFU in each tissue was determined. Bacterial colonization at 24 h postinfection was similar in all three tissues between wild-type-infected mice and ΔsfpA mutant-infected mice (data not shown). Bacterial loads in the Peyer’s patches and spleens were equivalent to those in wild-type-infected tissues at both 3 days and 7 days postinfection (Fig. 1A). However, by day 3 postinfection, a significantly smaller number of bacteria were recovered from the MLN of mice infected with the ΔsfpA strain (Fig. 1A). By day 7 postinfection, the number of CFU in the MLN of ΔsfpA strain-infected mice remained significantly lower (P = 0.0079) than that in wild-type-infected tissues, with
FIG. 1. Bacterial colonization of the mouse by the sfpA mutant. (A) C57BL/6j mice were orally infected with $10^8$ CFU wild-type Y. enterocolitica (JB580v) (■) or the sfpA mutant (YVM1051) (□). Mice were sacrificed and organs harvested on day 3 and day 7. The bacterial load for each organ was determined by counting the number of viable bacteria after plating serial dilutions. Results are expressed as CFU per gram of tissue. Each symbol represents an individual mouse, and symbols on the x axis were below the limits of detection (limits of detection were as follows: for Peyer's patches, 240; for MLN, 100; and for spleens, 65). The Mann-Whitney test was used to calculate the $P$ value. (B) C57BL/6j mice were infected intraperitoneally with $10^4$ CFU of wild-type Y. enterocolitica (JB580v) (■) or the sfpA mutant (YVM1051) (□). The Mann-Whitney test was used to calculate the $P$ value. Bacterial loads for mesenteric lymph nodes were determined as described above. (C) C57BL/6j mice were orally infected with $10^6$ CFU wild-type Y. enterocolitica (JB580v) (■), the sfpA mutant (YVM1051) (□), or the complemented sfpA mutant strain (YVM1220) (▴). Every “X” on the x axis represents one dead mouse; symbols on the x axis indicate that the CFU were below the limits of detection. P, Peyer's patch; M, mesenteric lymph node; S, spleen.
10 of the ΔsfpA strain-infected mice having no detectable CFU, in contrast to only 1 wild-type-infected mouse with no detectable CFU (limits of detection were as follows: for Peyer’s patches, 240 CFU; for MLN, 100 CFU; and for spleens, 65 CFU). These results suggest that SfpA is involved in the establishment, survival, and/or proliferation of *Y. enterocolitica* in the MLN.

The altered progression of infection to the MLN during oral infection with the ΔsfpA mutant suggested that this mutant was either defective in dissemination from the Peyer’s patches to the MLN or attenuated in its ability to survive in the MLN. To clarify the virulence defect of the ΔsfpA mutant, we determined the ability of the mutant to survive in the MLN independent of dissemination from the Peyer’s patches by inoculating mice via intraperitoneal injection, thus bypassing the Peyer’s patches altogether. C57BL6/j mice were injected with 10⁵ CFU of wild-type or ΔsfpA bacteria that were grown overnight at 26°C. At 24 hours postinfection, the MLN of all the mice infected with either strain had equivalent levels of colonization (data not shown). At 3 days postinfection, the MLN of all the mice infected with either strain had detectable numbers of bacteria; however, approximately 100-fold fewer bacteria were recovered from tissues of the ΔsfpA strain-infected animals (Fig. 1B). By 7 days postinoculation, bacteria were recovered from only 30% of the mice infected with the ΔsfpA mutant, in contrast to 80% of the wild-type-infected mice. The complementing strain, YVM1220, completely restored virulence and was able to colonize the MLN to wild-type levels (Fig. 1C). These results indicate that while the ΔsfpA mutant could establish infection in the MLN, it was attenuated in its ability to survive in the MLN and was cleared from this tissue at a much higher rate than that for wild-type bacteria. However, these results do not rule out an additional defect in dissemination by the mutant that would result in a smaller number of bacteria migrating from the Peyer’s patches to the MLN after oral infection.

**In vitro expression of SfpA.** SfpA was identified because it was expressed in the host but not in vitro at 26°C. Furthermore, Gort and Miller (8) demonstrated that the transcription of *sfpA* increases in vitro if bacteria are grown at 37°C. To determine the effect of transcriptional regulation on the overall SfpA protein levels in the cell, we generated a SfpAHA epitope-tagged gene fusion and introduced this into the chromosome of *Y. enterocolitica* by homologous recombination. Primers flanking the *sfpA* gene and primers to amplify two hemagglutinin (HA) tags were used to clone a copy of *sfpA* with two C-terminal HA tags into the suicide plasmid pSR47S (21). By homologous recombination, the native *sfpA* gene was replaced with *sfpA*ΔHA to generate strain YVM1219; substitution of *sfpA* with the fusion construct, *sfpA*ΔHA, was confirmed by Southern blotting and PCR (data not shown). To examine the expression of SfpAHA, broth cultures were grown at 26°C and 37°C, and samples were taken periodically. Total protein from each time point was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane (Schleicher and Schuell), and probed with anti-HA antibody (Sigma, St. Louis, MO) as described previously (26). The predicted molecular mass of SfpAHA with its signal sequence is 37 kDa, and that without the signal sequence is 35 kDa. As shown by transcriptional analysis, SfpAHA expression was absent at 26°C. At 37°C, SfpAHA levels peaked during early log phase (optical density at 600 nm [OD₆₀₀] = 0.5) and appeared to be degraded rapidly over time (Fig. 2). To determine the cellular localization of SfpA, YVM1219 was grown at 37°C overnight and then subcultured the next day for 2 hours. The cellular compartments were fractionated, and protein samples from whole-cell, cytoplasmic, periplasmic, and total membrane frac-
tions were analyzed by Western blotting (17). To ensure that proteins did not cross-contaminate fractions, Western blot analysis was performed using antibodies for known Y. enterocolitica cytoplasmic (RovA), periplasmic (β-lactamase), and membrane (Inv) proteins (data not shown). SfpAHA was only present in the whole-cell and membrane fractions (Fig. 3A) (17). Since the cellular fractionation results suggest that SfpA is a membrane protein, we wanted to determine if SfpA behaves similarly to other gram-negative outer membrane proteins. One hallmark of outer membrane proteins is that they are heat modifiable, referring to the differences in banding positions and numbers of bands for boiled versus unboiled samples (10, 34). Heat changes the conformation of numerous β-pleated sheets, resulting in altered electrophoretic mobility (15). SfpAHA in Y. enterocolitica showed different mobilities in boiled and unboiled samples, further suggesting that SfpA is an outer membrane protein (Fig. 3B).

SfpA appears to be a porin. A protein structure prediction program (http://bmerc-www.bu.edu/index.html) was used to further investigate SfpA (29, 33). In silico analysis of the structure of SfpA predicts the presence of 16 β-pleated sheets in the protein, suggesting the possibility that SfpA is a porin. β-Pleated sheets form β-barrels, which are the main structural components of outer membrane proteins, and porins often consist of β-barrels composed of 16 β-pleated sheets (27). In order to test the prediction that SfpA is a porin, sfpA was expressed in E. coli MCR106 and tested for growth on minimal medium in the presence of differently sized sugars. Overnight cultures grown in Luria-Bertani broth were pelleted, washed with M63 salts, and plated on minimal medium containing M63 salts, 10 μM IPTG, and 0.2% (wt/vol) of the indicated sugars. Growth was assessed after 24 to 48 h at 37°C.

FIG. 4. Sugar diffusion through the SfpA porin. sfpA (pSM1) was expressed in the E. coli MCR106 lamB mutant or in lamB⁺ E. coli strain MC4100 and tested for growth on minimal medium in the presence of differently sized sugars. Overnight cultures grown in Luria-Bertani broth were pelleted, washed with M63 salts, and plated on minimal medium containing M63 salts, 10 μM IPTG, and 0.2% (wt/vol) of the indicated sugars. Growth was assessed after 24 to 48 h at 37°C.
some that is involved in the colonization of MLN during acute
*Y. enterocolitica* infection. SfpA has amino acid sequence
similarity to hypothetical outer membrane proteins in three en-
Interestingly, there are no sfpA homologs in *Yersinia pestis*
or *Yersinia pseudotuberculosis*. While its exact mechanism in
virulence is unclear, there are several possible functions for
SfpA. Its activity as a porin within the nutrient-limited envi-
ronment of the host may promote the exchange of nutrients
and solutes. Alternatively, SfpA may have functions similar to
those of the *Neisseria gonorrhoeae* and *Neisseria meningitidis*
porins PorB1A and PorB1B, which induce apoptosis in host
eukaryotic cells (20, 28). Preliminary data suggest that when it is expressed in *E. coli*, SfpA does not act as an
adhesin for Chinese hamster ovary cells or confer serum
resistance (data not shown). Further studies will focus on
determining the mechanism by which SfpA affects the survival of
tuberculosis bacilli during systemic infection.

**Nucleotide sequence accession number.** The nucleotide se-
quence for *sfpA* has been deposited in GenBank under acce-
sion no. DQ470669.

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**Notes**

*Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia
receptors for invasion, a protein that promotes bacterial penetration into
solubility of outer membrane proteins of *Rhodopseudomonas sphaeroides*. J.
Cloning of the YenI restriction endonuclease and methyltransferase from
*Yersinia enterocolitica* serotype O8 and construction of a transformable
*Yersinia enterocolitica* porins ParA, B1A, and B1B, which induce apoptosis in host
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