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Characterization of a Novel Porin Involved in Systemic \textit{Yersinia enterocolitica} Infection

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Received 30 January 2006/Returned for modification 15 March 2006/Accepted 10 April 2006

\textit{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 $\Delta$sfpA strain is defective in mesenteric lymph node colonization. Bioinformatics and functional studies suggest that SfpA is a porin.

The genus \textit{Yersinia} includes three pathogenic species, \textit{Yersinia enterocolitica}, \textit{Yersinia pseudotuberculosis}, and \textit{Yersinia pestis}, which share a common tropism for lymphoid tissue. Upon ingestion, \textit{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 \textit{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 \textit{Y. enterocolitica} that are expressed during systemic infection (8). One gene identified in this screen, sif15, is not found in either \textit{Y. pseudotuberculosis} or \textit{Y. pestis}, and a strain carrying a mutation in sif15 was attenuated during intraperitoneal competitive infections with wild-type \textit{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 sif15 with the designation sfpA (systemic factor protein A) in order to avoid confusion with \textit{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 \textit{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 \textit{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 $\Delta$sfpA mutant was called strain YVM1051. Complementation of the $\Delta$sfpA 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 (pPutA). sfpA 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 $\Delta$sfpA mutation on virulence, C57BL/6j mice were orally infected with approximately $1 \times 10^6$ $\Delta$sfpA bacteria from an overnight culture grown in Luria-Bertani broth at 26°C and compared to mice receiving an analogous inoculation with wild-type \textit{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 $\Delta$sfpA 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 $\Delta$sfpA strain (Fig. 1A). By day 7 postinfection, the number of CFU in the MLN of $\Delta$sfpA 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 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 SfpHA 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 sfpAHA to generate strain YVM1219; substitution of sfpA with the fusion construct, sfpAHA, 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 gels and subjected to immunoblot analysis. (A) Y. enterocolitica sfpAHA cultures (YVM1219) were grown in Luria-Bertani broth at 26°C to an OD600 of 0.5 and fractionated as described above. Whole-cell lysates were boiled for 10 min in β-mercaptoethanol (BME) or Laemmli buffer (no BME) to rule out any effects of the BME. All samples were run in sodium dodecyl sulfate-polyacrylamide gels and subjected to immunoblot analysis with monoclonal anti-HA antibodies. The 35-kDa molecular mass marker is labeled. (B) The 35-kDa molecular mass marker is labeled.

FIG. 2. Levels of SfpAHA in Y. enterocolitica during in vitro growth. Cultures of Y. enterocolitica sfpA44A (YVM1219) were grown in Luria-Bertani broth at 26°C or 37°C, samples were collected at various time points, and aliquots with equivalent OD600 values were loaded into the lanes. Monoclonal anti-HA antibodies (Sigma, St. Louis, MO) were used in immunoblot analysis to determine the levels of SfpA.

FIG. 3. Localization and heat modifiability of SfpAHA in Y. enterocolitica. (A) Y. enterocolitica sfpA44A cultures (YVM1219) were grown in Luria-Bertani broth at 37°C to an OD600 of 0.5 and fractionated as described above. Whole-cell lysates were boiled for 10 min in β-mercaptoethanol (BME) or Laemmli buffer (no BME) to rule out any effects of the BME. All samples were run in sodium dodecyl sulfate-polyacrylamide gels and subjected to immunoblot analysis with monoclonal anti-HA antibodies. The 35-kDa molecular mass marker is labeled. (B) The 35-kDa molecular mass marker is labeled.
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 (H9252-β-lactamase), and membrane (Inv) proteins (data not shown). SfpA HA 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). SfpA HA 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 containing M63 salts, 10 μM IPTG, and 0.2% (wt/vol) of the indicated 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.

Conclusions. While the virulence plasmid of Y. enterocolitica is necessary for virulence, it is not sufficient, suggesting that there are important virulence factors encoded in the chromosome. sfpA is a novel virulence gene carried on the chromo-

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-
teric pathogens, namely, Salmonella enterica serovar Typhi, Escherichia coli O157:H7, and Helicobacter pylori (6, 25, 30). Interestingly, there are no sfpA homologous 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 in host cells, act as adhesins for Chinese hamster ovary cells or confer serum 
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 
cells, act as adjuvants to activate the immune system, and aid 
in the invasion of epithelial cells (2, 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 de-
termining the mechanism by which SfpA affects the survival of 
bacteria during systemic infection.

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

We thank Joseph W. St. Gene III for his experimental and technical advice and Matthew Lawrenz, Chris O’Connor, Clemencia Rojas, and Kim Walker for their critical reviews of the manuscript prior to sub-
mission. This work was supported by research grant AI42736 (V.L.M.).

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