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Functional Conservation among Members of the *Salmonella typhimurium* InvA Family of Proteins

CHRISTINE C. GINOCCHIO† AND JORGE E. GALÁN*

Department of Molecular Genetics and Microbiology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-5222

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InvA, which is essential for *Salmonella* spp. to enter cultured epithelial cells, is a member of a family of proteins involved in either flagellar biosynthesis or the secretion of virulence determinants by a number of plant and mammalian pathogens. The predicted overall secondary structures of these proteins show significant similarities and indicate a modular construction with a hydrophobic amino-terminal half, consisting of six to eight potential transmembrane domains, and a hydrophilic carboxy terminus which is predicted to reside in the cytoplasm. These proteins can be aligned over the entire length of their polypeptide sequences, with the highest degree of homology found in the amino terminus and clusters of conserved residues in the carboxy terminus. We examined the functional conservation among members of this protein family by assessing the ability of MxiA of *Shigella flexneri* and LcrD of *Yersinia pseudotuberculosis* to restore invasiveness to an *invA* mutant of *Salmonella typhimurium*. We found that MxiA was able to complement the entry defect of the *invA* mutant strain of *S. typhimurium*. In contrast, LcrD failed to complement the same strain. However, a plasmid carrying a gene encoding a chimeric protein consisting of the amino terminus of LcrD and the carboxy terminus of InvA complemented the defect of the *Salmonella invA* mutant. These results indicate that the secretory systems in which these proteins participate are functionally similar and that the *Salmonella* and *Shigella* systems are very closely related. These data also suggest that determinants of specificity may be located at the carboxy termini of these proteins.

The pathogenicity of *Salmonella* spp. is dependent upon the ability of these organisms to gain access to cells that are normally nonphagocytic. We have previously characterized a chromosomally encoded locus, *inv*, which is essential for *Salmonella* entry into cultured epithelial cells (5). Nonpolar mutations in *invA*, one of the 15 genes thus far identified in the *inv* locus, significantly (~500-fold) reduced the ability of *Salmonella* spp. to enter cultured epithelial cells, without significantly affecting the levels of attachment to the same cells (7). This is consistent with the observation that *invA* mutant strains of *Salmonella typhimurium* showed reduced virulence when administered orally to mice, presumably because of their inability to gain access to the intestinal epithelium (5). In addition, *invA* is present and functional in most, if not all, virulent *Salmonella* strains (6).

The InvA protein has a predicted molecular weight of 71,000, with a secondary structure consistent with that of a polytopic integral membrane protein composed of two domains: a hydrophobic amino-terminal half consisting of eight putative membrane-spanning segments and a hydrophilic carboxy-terminal region which most likely resides in the cytoplasm (7). This distinct modular construction and the fact that each domain resides in a different cellular compartment suggest that the amino and carboxy termini of InvA may provide different functions to the protein.

A comparison of the predicted sequence of InvA with translated sequences in GenBank (release 79) revealed significant homology to a family of proteins that are components of sec-independent protein secretion systems, now designated as type III secretory systems. One class of these proteins, including MxiA of *Shigella* spp. (1, 17), LcrD of *Yersinia* spp. (19), HrpO of *Pseudomonas solanacearum* (9), HrpI of *Erwinia amylovora* (27) and *Pseudomonas syringae* (13), and HrpC2 of *Xanthomonas campestris* (4), is involved in the surface presentation or secretion of virulence determinants. A second class of these proteins, which includes FlbF of *Caulobacter crescentus* (20), FlhA of *Escherichia coli* (15) and *Bacillus subtilis* (2), and FlsA of *Campylobacter jejuni* (18) and *Helicobacter pylori* (18), is required for flagellar biosynthesis.

All members of this protein family can be aligned over the entire length of the protein sequences, although the highest degree of homology is found in the hydrophobic amino-terminal half (7). There are, however, clusters of highly conserved residues in the carboxy terminus, and the overall secondary structures of these proteins show significant similarity. The similarity in the structural features and the proposed functions of these proteins suggest that their domains may have evolved from a common ancestor and that they may provide similar functions in the different systems. The availability of a family of proteins from different organisms performing similar functions provides a powerful tool to carry out structure-function analysis, which may help in understanding the role of InvA in bacterial entry.

We examined the functional conservation between InvA and its cognate proteins, MxiA of *Shigella flexneri* and LcrD of *Yersinia pseudotuberculosis*, by comparing their ability to complement an *invA* nonpolar mutation in *S. typhimurium*. The *mxiA* and *lcrD* genes were cloned by PCR, from the wild-type strains *S. flexneri* M90T (22) and *Y. pseudotuberculosis* YPIII (8), respectively, by using a commercial kit according to the instructions of the manufacturer (GeneAmp, Perkin-Elmer Cetus, Norwalk, Conn.) and standard recombinant DNA techniques (16). Expression of the cloned genes was placed under

* Corresponding author. Phone: (516) 632-8780. Fax: (516) 63208891. Electronic mail address: galan@asterix.bio.sunysb.edu.

† Present address: Department of Infectious Diseases, North Shore University Hospital—Cornell Medical College, Manhasset, NY 11030.

the control of the *invA* promoter by creating in-frame fusions between the homologous genes (*mxiA* and *lcrD*) and the first 22 codons of *invA* and its upstream promoter region. The presence of the *invA* putative promoter and ribosomal binding site sequences ensured the proper transcription and translation of the *Shigella mxiA* and *Yersinia lcrD* genes in *S. typhimurium.*

The *mxiA* gene was cloned as follows. A primer (5′-GGTTA TGATCATTGCTATGTTCGTCA-3′) derived from the *mxiA* sequence (1) was used to change nucleotides 84 and 85 of the *mxiA* coding sequence from a G to a C, and from an A to a G, respectively, thus removing the second of the two *Bcl*I restriction sites present in this coding sequence. A second primer (5′-CGTGTTACTGATATCTAAACCTCACTA-3′) derived from the *mxiA* sequence was used to generate a unique *Eco*RV site after the stop codon of *mxiA*. With *S. flexneri* M90T total cell DNA used as a template, these two primers were used to amplify by PCR a 2.0-kb DNA fragment containing the *mxiA* gene (1). The 2.0-kb amplification product was excised from a 0.7% agarose gel with GeneClean (Bio101, La Jolla, Calif.), kinased according to standard protocols with T4 polynucleotide kinase (BRL, Gaithersburg, Md.), and blunt-end cloned into the *Eco*RV site of pBluescript-SKII$^+$, to generate pSB153. A *Bcl*I-*Eco*RV fragment of pSB150 containing *invA* under the control of the T7 promoter (7) was replaced by the equivalent fragment of pSB153 encoding *mxiA*. The resulting plasmid, pSB154, contains the *mxiA* gene, minus the first 22 codons, cloned in frame to the upstream promoter regions and the first 22 amino-terminal codons of *invA*.

To clone *lcrD*, a primer (5′-GCTGCTGTTAGCTGATATC TTCATGATGGT-3′), derived from the *lcrD* sequence (19), was used to create a unique *Eco*RV site at nucleotides 78 to 83 of the *lcrD* coding sequence. This new site allows an in-frame fusion of *lcrD* to the *Bcl*I site of *invA*, after the ends are filled in with the Klenow fragment of DNA polymerase I (BRL). A second primer (5′-CGGTAAGCCACGGAATTCAAAGGTC T-3′) generated an unique *Eco*RI restriction site after the stop codon of *lcrD*. These two primers were used to amplify, by PCR, a 2.0-kb DNA fragment from the *Y. pseudotuberculosis* YPIII virulence plasmid which contains the *lcrD* gene (8). The 2.0-kb amplification product was excised from a 0.7% agarose gel with GeneClean, digested with *Eco*RV and *Eco*RI, and inserted into the *Eco*RV/*Eco*RI sites of pBluescript-SKII$^+$ to generate pSB158. The *lcrD* gene was then retrieved from pSB158 by digesting with *Eco*RI and *Eco*RV and cloned into the *Eco*RI and *Bcl*I sites of pSB150, after treatment with the Klenow fragment of DNA polymerase I, yielding the plasmid pSB159. This plasmid contains *lcrD*, minus the first 27 codons, cloned in frame to the upstream promoter regions and the first 22 amino-terminal codons of *invA*.

To confirm that the plasmid constructions generated in-frame fusions resulting in chimeric proteins of the proper size, pSB150, pSB154, and pSB159, encoding *invA*, *mxiA*, and *lcrD*, respectively, were transformed into *E. coli* BL21(DE3), a strain that carries the T7 RNA polymerase gene under the control of p*lac* (24). Upon induction with IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma, St. Louis, Mo.), plasmid-encoded proteins were selectively labeled with ^{35}S-methionine as described previously (25). As shown in Fig. 1, a polypeptide with an estimated molecular weight of ~70,000 was observed in each lysate of strains carrying pSB150 (lane B), pSB154 (lane C), and pSB159 (lane D) but was absent from the strain carrying the vector pBluescript-SKII$^+$ alone (lane A). These data indicate that pSB150, pSB154, and pSB159 were capable of expressing chimeric proteins of the appropriate size, as determined by sequence analysis.

By using an in vitro tissue culture assay previously described (5), MxiA and LcrD were tested for their ability to complement the entry defect of SB147, a strain of *S. typhimurium* SR11 which contains a nonpolar mutation in the *invA* gene (7). As shown in Table 1, pSB154, encoding *mxiA*, was capable of restoring the entry phenotype of SB147 to about half of the level of the positive control plasmid pSB150, which carries a wild-type copy of *invA*. On the other hand, the introduction of pSB159, encoding *lcrD*, into SB147 did not complement the entry deficiency of this strain.

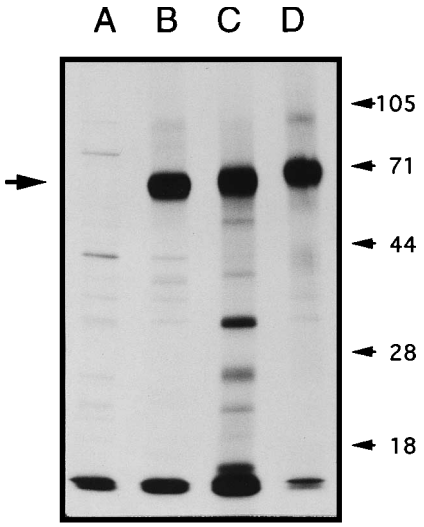

FIG. 1. Expression of *invA*, *mxiA*, and *lcrD* using a T7 promoter-polymerase system. The *invA*, *mxiA*, and *lcrD* genes were placed downstream from the bacteriophage T7 promoter of pBluescript-SKII$^+$ and introduced into *E. coli* BL21(DE3), which carries the bacteriophage T7 RNA polymerase gene under the control of p*lac* (24). Upon induction of the RNA polymerase gene, plasmid encoded proteins were selectively labeled with ^{35}S-methionine and whole-cell lysates were separated on a sodium dodecyl sulfate-polyacrylamide gel. Lanes: A, pBluescript-SKII$^+$ (vector); B, pSB150 (*invA*); C, pSB154 (*mxiA*); D, pSB159 (*lcrD*). Numbers to the right denote the positions of the molecular mass standards (in kilodaltons). The arrow to the left denotes the position of the InvA, MxiA, and LcrD proteins.

Since LcrD was not capable of functionally substituting for InvA, we reasoned that the construction of chimeric proteins consisting of different combinations of domains of InvA and LcrD may allow the identification of regions of InvA which confer functional species specificity to this protein. Since the amino termini of InvA and LcrD were highly conserved and the carboxy termini were more divergent, we constructed two chimeric proteins in which we replaced either the carboxy terminus of LcrD or the carboxy terminus and the last three

TABLE 1. Complementation of an InvA$^-$ strain of *S. typhimurium* by MxiA of *S. flexneri* and LcrD of *Y. pseudotuberculosis*

Strain (relevant genotype)	Complementing plasmid	Plasmid-encoded protein	% Internalization[a]
SR11 (wild type)	None		90.04 ± 2.4
SB147 (*invA*)	None		0.05 ± 0.005
SB147 (*invA*)	pSB150	InvA	65.66 ± 2.49
SB147 (*invA*)	pSB154	MxiA	26.01 ± 1.77
SB147 (*invA*)	pSB159	LcrD	0.032 ± 0.006

[a] Internalization is expressed as the percentage of the inoculum of bacteria that was insensitive to gentamicin treatment because of cell invasion. Values are means ± standard deviation of triplicate samples. Similar results were obtained in several repetitions of this experiment.

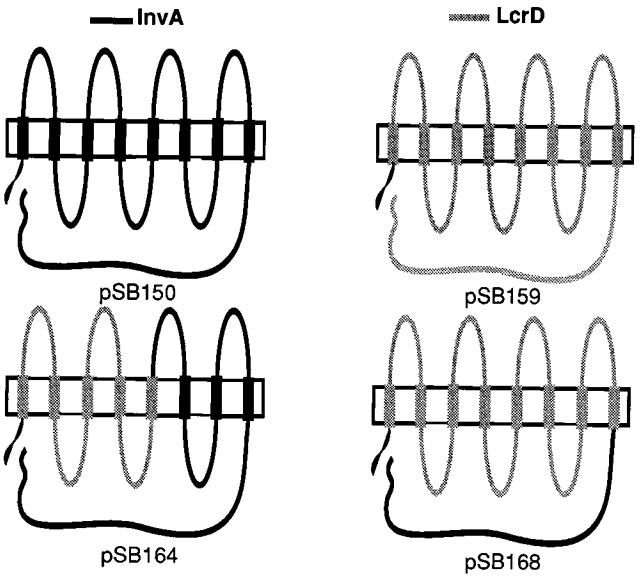

FIG. 2. Schematic representation of the LcrD-InvA chimeric proteins expressed by the different plasmids.

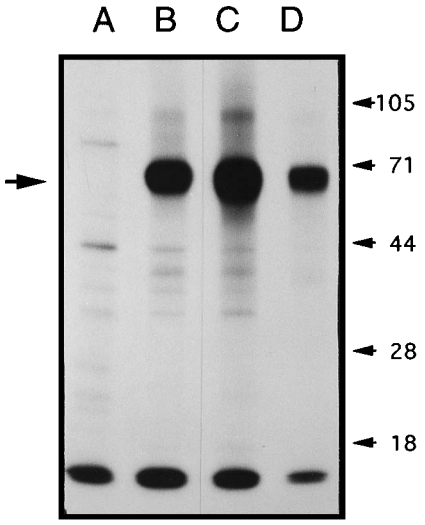

FIG. 3. Expression of *lcrD*/*invA* gene fusions by using a T7 promoter-polymerase system. The *lcrD*/*invA* gene fusions were placed downstream from the bacteriophage T7 promoter of pBluescript-SKII$^+$ and introduced into *E. coli* BL21(DE3), which carries the bacteriophage T7 RNA polymerase gene under the control of p*lac* (20). Upon induction of the RNA polymerase gene, plasmid-encoded proteins were selectively labeled with ^{35}S-methionine and whole-cell lysates were separated on a sodium dodecyl sulfate-polyacrylamide gel. Lanes: A, pBluescript-SKII$^+$ (vector); B, pSB150; C, pSB164; D, pSB168. Numbers to the right denote the positions of the molecular mass standards (in kilodaltons). The arrow to the left denotes the position of the InvA and LcrD/InvA chimeric proteins.

membrane-spanning regions of LcrD with comparable regions of InvA.

The LcrD/InvA chimeric proteins were constructed as follows. With pSB159 as a template and site-directed mutagenesis (14), the oligonucleotide 5′-GGTGTTACCCACCATGGATT AGCGG-3′ was used to generate a unique *Nco*I site at codons 221 and 222 of *lcrD*, yielding pSB163. This site is equivalent to a unique *Nco*I site at codons 218 and 219 of *invA* which correspond to the predicted periplasmic loop between the fifth and sixth transmembrane regions of the encoded InvA. Plasmid pSB163 was digested with *Nco*I and *Eco*RI, and the corresponding *Nco*I-*Eco*RI fragment of *invA* was cloned into these sites to generate pSB164. pSB164 contains the upstream promoter sequences and the coding sequence for the first 22 amino-terminal residues of InvA, the first five transmembrane domains of LcrD, and the final three transmembrane domains and the whole carboxy terminus of InvA (Fig. 2).

The second chimeric construct was generated as follows. With pSB159 as a template, the nucleotide 5′-GGCTGCTC GATCGCGAGCCAAAACAAG-3′ was used to generate, by site-directed mutagenesis, the plasmid pSB165, which contains a new unique *Nru*I site at codons 349 and 350 of the LcrD coding sequence and is equivalent to codons 337 and 338 of the InvA coding sequence. The *Nru*I site is located two codons past the end of the coding sequence of the eight putative transmembrane domains of LcrD. With the mutagenic nucleotide 5′-CAGTATTGAGTCGCGAGAAGGGTC-3′ and pSB150 as a template, a comparable unique *Nru*I site was also generated in *invA* at codons 340 and 341, located 23 codons after the coding sequence of the last predicted membrane-spanning region in InvA. The 3′ half of *invA* was retrieved from the resulting plasmid, pSB167, by digesting with *Nru*I and *Eco*RI and cloned into the *Nru*I-*Eco*RI sites of pSB165 to generate pSB168. This plasmid contains the upstream promoter sequences and the coding regions for the first 22 amino-terminal residues of InvA, the amino-terminal eight transmembrane domains of LcrD, and the carboxy terminus of InvA (Fig. 2).

As shown in Fig. 3, both pSB164 and pSB168 were able to express chimeric proteins of the proper sizes. When tested for their ability to complement the entry defect of SB147, both pSB164 and pSB168 were able to significantly restore invasiveness to an *invA* mutant strain of *S. typhimurium* (Table 2). The level of complementation with pSB164 was slightly higher than that achieved with pSB168, presumably because of the presence of additional *invA*-specific sequences.

Among the homologous type III secretory systems, *Salmonella inv* gene products share the highest degree of similarity with the *spa* and *mxi* gene products of *Shigella* spp. These loci are required for the surface presentation of the Ipa proteins which are necessary for *Shigella* spp. entry into cultured epithelial cells (12, 26). This high degree of homology is consistent with the high level of complementation of an *invA* mutant of *S. typhimurium* by MxiA of *S. flexneri*. The functional homology among the *Salmonella* and *Shigella* secretory systems is further supported by the findings of Groisman and Ochman, who reported the complementation of *spaP* (*invL*) mutant of *S. typhimurium* with its *Shigella* homolog *spa24* (10). Invasion studies

TABLE 2. Complementation of an InvA$^-$ strain of *S. typhimurium* by LcrD/InvA chimeric proteins

Strain (relevant genotype)	Complementing plasmid	Plasmid-encoded protein[a]	% Internalization[b]
SR11 (wild type)	None		90.04 ± 2.4
SB147 (*invA*)	None		0.05 ± 0.005
SB147 (*invA*)	pSB150	InvA	65.66 ± 2.49
SB147 (*invA*)	pSB159	LcrD	0.032 ± 0.006
SB147 (*invA*)	pSB164	LcrD/InvA	35.202 ± 1.8
SB147 (*invA*)	pSB168	LcrD/InvA	11.45 ± 0.2

[a] See Fig. 2.
[b] Internalization is expressed as the percent of the inoculum of bacteria that were insensitive to gentamicin treatment because of cell invasion. Values are means ± standard deviation of triplicate samples. Similar results were obtained in several repetitions of this experiment.

examining the ability of other *Shigella* proteins to complement other *Salmonella inv* mutants could determine the extent to which components of these secretory systems are exchangeable.

LcrD is necessary for the secretion and transcriptional regulation of a number of *Yersinia* outer membrane proteins (Yops) and V antigen which, as part of the low calcium response, are necessary for this organism's display of virulence and host invasion (23). The observation that LcrD was not able to complement an *invA* mutant of *S. typhimurium* suggests that although InvA, MxiA, and LcrD may have a common function, the *Yersinia* secretory system and/or its regulation are more divergent from those of *Shigella* spp. and *Salmonella* spp. The ability of the LcrD/InvA chimeric proteins to complement the entry defect of SB147 indicates that the amino termini of these proteins are interchangeable and functionally similar. This region may serve as a membrane anchor or may function in the formation of a pore through which secreted virulence determinants are translocated through the inner membrane. Our results also suggest that regions in the carboxy termini of these proteins may determine specificity for each of the secretory systems. These regions may contain unique domains which are recognized by and allow the interactions of the various target proteins which compose each secretory apparatus. The construction of additional chimeric proteins, interchanging various domains and regions within each domain, may determine not only regions of specificity for each secretory system but also functional domains of the protein.

Various targets of these type III secretory systems, including the Ipas of *Shigella* spp. (21), the Yops of *Yersinia* spp. (23), and the harpins (11, 27, 28) from a number of plant pathogens, have been identified. Recently, we have reported the identification of a secreted protein, InvJ, which is necessary for the entry of *S. typhimurium* into cultured epithelial cells and whose secretion is dependent upon various members of the *inv* locus (3). The identification of this secreted virulence determinant has now provided us with a valuable tool to further elucidate the role of InvA and other Inv proteins in what appears to be a similar process, that is, the surface presentation and/or secretion of virulence proteins which lack a typical prokaryotic signal sequence.

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