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Identification of Genes Affecting *Salmonella enterica* Serovar Enteritidis Infection of Chicken Macrophages

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Screening of 7,680 *Salmonella enterica* serovar Enteritidis mutants for attenuation in a chicken macrophage infection model yielded a series of mutants including several with defects in previously unrecognized *Salmonella* virulence genes. One of the newly identified genes was the *pbpA2* gene, belonging to the penicillin binding protein gene family.

Salmonella strains pose a major problem in public health, causing diseases ranging from gastroenteritis to typhoid fever. In recent years, *Salmonella enterica* serovar Enteritidis has replaced serovar Typhimurium as the primary etiologic agent of *Salmonella* infections in many countries (15). A likely source of serovar Enteritidis is the consumption of infected poultry (6, 15). The molecular mechanisms that enable this serovar to persist in poultry are poorly defined. To address this topic, we generated a mini-Tn*10* mutant library of serovar Enteritidis strain CVI-1 and searched for cellular-infection-impaired mutants in a chicken macrophage infection model.

The mutant library was constructed by transforming strain CVI-1 with plasmid pKO3 carrying the mini-Tn*10* delivery system from plasmid pLOF/KM (7). Induction of the transposition event eventually yielded 7,680 kanamycin-resistant colonies that were individually collected and tested for their ability to invade or survive in the chicken macrophage HD-11 cell line (5). After several rounds of selection which involved plate counting of the number of intracellular bacteria recovered after 30 min of infection and 2 h of treatment with colistin (100 μg/ml) to kill the extracellular microorganisms, 37 clones (designated SEM1 to SEM37) were identified that consistently yielded reduced (5 to 50% of wild-type) levels of cellular infection.

Genetic characterization of the selected mutants with inverse PCR using outward-oriented transposon primers followed by DNA sequencing of the products revealed the transposon insertion sites for 36 of the 37 mutants (Table 1). Sequence analysis indicated that in 14 strains (38%, group I in Table 1) genes had been disrupted that shared homology with genes involved in the assembly of flagella or bacterial motility in serovar Typhimurium. Analysis of the 37 selected mutants for their behavior in U-shaped tubes containing 0.4% Luria-Bertani soft agar demonstrated that all mutants in group I indeed were nonmotile. Bacterial motility has previously been demonstrated to expedite *Salmonella* entry into cultured epithelial cells, probably by enabling more rapid contact with the monolayer (13, 21). Fluorescence-activated cell sorting analysis with the serovar Enteritidis strain CVI-1 and the mutant strains made fluorescent by introduction of the plasmid pEGFP, encoding the green fluorescent protein, yielded similar results for chicken macrophages (data not shown), explaining the organisms' poor recovery from the cells.

From the group of motile mutants (group II in Table 1), three carried the transposon in homologues of *invG*, *invI*, and *spaS* located in *Salmonella* pathogenicity island 1 (SPI-1). Six mutants had defects in genes involved in lipopolysaccharide (LPS) biosynthesis (*rfaI*, *rfaJ*, and *rfbI*) or lipid A modification (*pmrB* and *pmrF*), four had defects in homologues of global regulatory genes (*phoP*, *oxyR*, and *barA*), one had a defect in a peptidoglycan synthesis gene (*pbpA*), seven had defects in putative metabolic genes (*ugd*, *tdh*, *icd*, and *cat2*), and one had a defect in a homologue of the *Escherichia coli yegQ* gene. For serovar Typhimurium, SPI-1, which encodes a type III secretion system, and LPS have been demonstrated previously to facilitate bacterial invasion of mammalian cells (10) and intracellular survival (8, 11, 12), respectively. Similarly, the regulator proteins PhoP (8), OxyR (18), and BarA (2) contribute to serovar Typhimurium virulence. These data confirm the existence of considerable functional conservation of these pathogenic mechanisms among the serovars Enteritidis and Typhimurium and indicate that these mechanisms are also important in infection of chicken macrophages.

Several of the metabolic genes defective in our mutants have not been previously identified as *Salmonella* virulence determinants. Apart from the PhoP/PhoQ-regulated *ugd* (*pagA*) gene, which in serovar Typhimurium has been demonstrated to be transcriptionally active inside macrophages and to be necessary for growth in a low-magnesium environment (1, 19, 20), two genes were identified with homology to the *E. coli tdh* gene and the *cat2* gene in *Clostridium aminobutyricum*. These genes encode a threonine dehydrogenase (3) and a 4-hydroxybutyrate coenzyme A transferase (16), respectively. In *E. coli*, the *tdh* homologue is regulated by the *lrp* gene (9), which is one of the regulators of virulence genes in serovar Typhimurium (14). The function of the *tdh* gene in *Salmonella* has never been established. An additional previously unrecognized gene important for cellular infection of chicken macrophages was the serovar Enteritidis *icd* gene, which may encode the enzyme

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TABLE 1. Identification and characterization of serovar Enteritidis mutants impaired in their ability to invade and/or survive in chicken macrophages

Group[a]	Serovar Enteritidis mutant(s)	BLAST identity[b] (%)	Insertion locus	Putative function
I	7, 14, 15, 16	86 X	*fliD*	Flagellar hook-associated protein 2
	5, 21, 28	98	*flhD*	Transcriptional activator for motility genes
	33	98	*flhC*	Transcriptional activator for motility genes
	36	97	*fliA*	Flagellum-specific promoter
	25	98	*fliL*	Flagellar structure protein
	41	97	*fliN*	Flagellar switch protein
	38	98	*fliP*	Unknown
	43	94	*motA*	Flagellar motor protein
	37	94	*flgI*	Flagellar Pring
II	6	94	*invG*	SPI-1, type III secretion system
	13	98	*invI*	SPI-1, type III secretion system
	27	90	*spaS*	SPI-1, type III secretion system
	8	95	*rfbI*	LPS side chain synthesis
	42	91	*rfaI*	LPS outer core synthesis
	2	97	*rfaJ*	LPS outer core synthesis
	1	98	*pmrB*	Regulator of *pmr* gene expression
	9, 30	92	*pmrF*	Lipid A modification
	19, 24	99	*phoP*	Regulator of virulence genes
	44	84	*oxyR*	Sensor and regulator of oxidative stress
	12	100	*barA*	Sensor and regulator
	35	97	*pbpA2*	Peptidoglycan synthesis
	10, 11, 17, 23	97	*ugd*	UDP-glucose dehydrogenase
	20	92	*tdh*	Threonine dehydrogenase
	31	36	*cat2*	4-Hydroxybutyrate coenzyme A transferase
	3	90	*icd*	Isocitrate dehydrogenase
	26	81	*yegQ*	Putative protease precursor
	29[c]			

[a] Group I are nonmotile mutants; group II are motile mutants.
[b] Percentages of sequence identity were obtained by BLASTN analysis of obtained sequence flanking the mini-Tn*10*. X indicates that the BLASTX algorithm instead of BLASTN was used.
[c] For mutant 29, only plasmid sequence was obtained, suggesting that in this mutant the vector carrying mini-Tn*10* may have been integrated into the genome.

isocitrate dehydrogenase. This gene is considered to have a housekeeping function and appears to be conserved among most *Salmonella* serovars (22). Together, these results strongly suggest that metabolic adaptation is an important feature in serovar Enteritidis infection of chicken macrophages. Whether the newly identified genes also contribute to virulence of other *Salmonella* serovars and/or in other hosts awaits further study.

Another gene not previously implicated in *Salmonella* virulence was the *pbpA2* gene defective in mutant SEM35. This gene exhibited approximately 60% homology with *E. coli pbpA* encoding penicillin binding protein 2 (4). Analysis of the serovar Typhimurium genome sequence indicated that this serovar carries two related *pbpA* sequences, one (designated *pbpA*) positioned adjacent to the putative *rodA* locus, and the other (designated *pbpA2*) flanking the *argS* locus. The latter gene was >99% identical in sequence to the gene disrupted in SEM35. To ascertain that the mutant phenotype of SEM35 was caused by disruption of *pbpA2*, intact copies of both the serovar Enteritidis *pbpA* and *pbpA2* genes were PCR amplified, cloned onto plasmid pWSK29, and introduced into SEM35. Infection assays with HD-11 cells demonstrated that

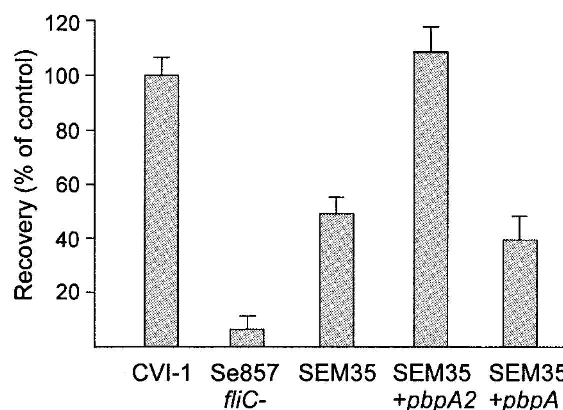

FIG. 1. Recovery of strains CVI-1 (positive control), Se857*fliC* mutant (nonflagellated, negative control) (21), SEM35 (*pbpA2* mutant), and SEM35 carrying pWSK29::*pbpA2* or SEM35 carrying pWSK29::*pbpA*, from the intracellular compartment of HD-11 macrophages after 30 min of infection and 2 h of colistin treatment. Data are the means ± standard deviations of three experiments.

the intact *pbpA2* gene completely restored the wild-type phenotype, while the *pbpA* gene was unable to complement the defect in SEM35 (Fig. 1). In serovar Typhimurium, the promoter of the *pbpA2* gene is acid inducible (20), which could point to a role in intracellular survival. Furthermore, the homology of PbpA2 with the members of the penicillin binding protein family may indicate a role in cell wall synthesis and in the resistance against antimicrobial peptides (17). Thus, PbpA2 may contribute to intracellular survival of *Salmonella* by providing resistance to antimicrobial peptides in a low-pH environment. The finding that the *pbpA* gene was unable to complement *pbpA2* function indicates that these two related genes have different functional properties.

Thus far, the molecular mechanisms that enable serovar Enteritidis to colonize and infect chicken macrophages are largely unknown and assumed to resemble those of the related serovar Typhimurium. Our experimental work largely confirms this notion, although a number of novel genes involved in cellular infection of chicken macrophages were identified. It can be expected that further detailed analysis of the isolated mutants will shed more light on the possible host, cell type, and/or pathogen specificity of these bacterial traits and their potential as targets for future infection intervention.

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