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-Tn10 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-Tn10 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 *Salmonella* genes involved in flagellar assembly or bacterial motility. Apart from the PhoP/PhoQ-regulated *ugd* gene, which in *Salmonella* has been demonstrated to be transcriptionally active inside macrophages and to be necessary for growth in a low-magnesium environment (1, 19, 20), 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 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.
isocitrate dehydrogenase. This gene is considered to have a housekeeping function and appears to be conserved among Salmonella genes also contributing to virulence of other serovars (22). Together, these results strongly suggest that metabolic adaptation is an important feature in the newly identified gene defective in mutant SEM35. This 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 pbpA 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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REFERENCES


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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**

<table>
<thead>
<tr>
<th>Group</th>
<th>Serovar Enteritidis mutant(s)</th>
<th>BLASTP identity (%)</th>
<th>Insertion loci</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7, 14, 15, 16, 29</td>
<td>86</td>
<td>fliD</td>
<td>Flagellar hook-associated protein 2</td>
</tr>
<tr>
<td>II</td>
<td>6, 12, 19, 24, 26</td>
<td>97</td>
<td>invG</td>
<td>SPI-1, type III secretion system</td>
</tr>
<tr>
<td></td>
<td>27</td>
<td>96</td>
<td>pmrF</td>
<td>Lipid A modification</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>95</td>
<td>ptsP</td>
<td>Regulator of virulence genes</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>94</td>
<td>oxyR</td>
<td>Sensor and regulator of oxidative stress</td>
</tr>
<tr>
<td></td>
<td>12, 15</td>
<td>100</td>
<td>barA</td>
<td>Sensor and regulator</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>97</td>
<td>tdi</td>
<td>Threonine dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>97</td>
<td>cat2</td>
<td>4-Hydroxybutyrate dehydrogenase</td>
</tr>
<tr>
<td></td>
<td>9, 29</td>
<td>90</td>
<td>icd</td>
<td>Isocitrate dehydrogenase</td>
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<tr>
<td></td>
<td>26</td>
<td>81</td>
<td>vegQ</td>
<td>Putative protease precursor</td>
</tr>
</tbody>
</table>

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* Group I are nonmotile mutants; group II are motile mutants.

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