Signature-Tagged Mutagenesis to Identify Virulence Genes in Salmonella choleraesuis

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Abstract

Signature-tagged mutagenesis is a functional genomics approach to identify bacterial virulence genes by simultaneously screening multiple mutants in a single host animal. Avirulent (attenuated) mutants are identified by negative selection (failure to colonize the host). The method was recently developed to investigate Salmonella typhimurium in a mouse model of human typhoid fever. We modified the protocol to investigate virulence genes of S. choleraesuis in its natural host, the pig. First, we generated random, knock-out (null) mutations in S. choleraesuis using transposon-mediated insertion of unique, signature-tagged (40 bp), kanamycin resistance cassettes. To validate the modified protocol, a test pool of 45 mutants was inoculated orally or intraperitoneally (systemic infection) into pigs. Three of the mutants were not identified from cultures of the mesenteric lymph nodes obtained four days after infection. Attenuation of these three candidate attenuated mutants was confirmed by mixed challenge growth experiments (growth of a 1:1 mixture of mutant and wild type S. choleraesuis) in broth cultures and in pigs. All three mutants were attenuated for infection of pigs as the mutants were at a marked disadvantage relative to the wild type bacteria for growth. For one mutant, the competitive indices (ratios of mutant to wild type bacteria) were 0.15 and 0.03 for bacteria in the intestines and mesenteric lymph nodes, respectively; for the other two mutants, the indices were about 0.001 in either tissue. In contrast, none of the mutants were at a growth disadvantage in broth cultures. As an additional control, two random mutants recovered from the pigs inoculated with the pool (and therefore not candidates for attenuation) were also tested in the mixed challenge experiments. As expected, the competitive growths of these mutants in culture medium and in pigs were similar to that of the wild type. In one of the three attenuated mutants the inactivated gene has been identified, after cloning and sequencing, as hilA. In mouse models of S. typhimurium infection, hilA is associated with enteric invasion, but not systemic proliferation. Because the hilA mutant failed to colonize pigs irrespective of oral or systemic inoculation, its function in pigs is likely to be more complex than in the mouse. Our data show signature-tagged mutagenesis can be used to identify Salmonella genes associated with virulence in pigs. As we continue screening mutants, we expect to identify novel genes related to pathogenicity and we are expanding our studies to include S. typhimurium.

Introduction

Salmonella choleraesuis causes over 95% of all swine salmonelloses and, although host-adapted to pigs, occasionally causes a severe form of foodborne salmonellosis in humans (Schwartz, 1999). The reservoir for S. choleraesuis is the pig intestine. Disease typically involves systemic invasion. Little is known about the mechanisms (factors) involved in disease pathogenesis (Gray & Fedorka-Cray, 1996). Bacterial pathogens typically have multiple virulence factors; for example, at least 60 genes have a role in S. typhimurium infections of mice (Groisman & Ochman, 1997). Sequencing the genome of a pathogen will predict relatively few and confirm none of the virulence factors. An efficient, direct method to investigate virulence factors is to use functional genomics. A functional genomics method was recently developed using signature-tagged transposon mutagenesis to investigate S. typhimurium in mice as a model of human typhoid fever (Hensel et al., 1995; Holden & Hensel, 1998; Perry, 1999). Virulence genes of bacteria that infect multiple hosts species are not necessarily the same among different potential hosts (Tsolis et al., 1999). We have adapted the
signature tag protocol to scan *S. choleraesuis* for virulence genes important for infecting its natural host, the pig.

**Goal**

To adapt the signature-tagged mutagenesis protocol to identify genes of *Salmonella choleraesuis* important for virulence (pathogenicity) in pigs.

**Material and Methods**

![Diagram of the signature-tagged mutagenesis protocol](image)

**Figure 1.** Signature-tagged mutagenesis screening protocol for *Salmonella* pathogenicity genes in swine. The protocol is a negative selection method to scan the bacterial genome for genes which, when inactivated, cause bacteria to lose their ability to colonize host pigs. Mutants have a random gene inactivated by the insertion of a signature-tagged cassette. The signature tags are unique 40 bp double-stranded oligonucleotides. The Recovery pool (*Salmonella* isolated from inoculated pigs) is compared to the Input pool (inoculum) by colony hybridization probing with labeled signature tags generated by PCR amplification. If the mutant is in the Input pool but not the Recovery pool, the gene tagged in that mutant is a candidate for having a role in pathogenesis. The half arrowheads indicate invariant PCR primers; the primer sequence is excised from the labeled PCR amplicon before probing.
Our protocol to identify virulence genes of *S. choleraesuis* by signature-tagged mutagenesis is diagramed in Figure 1. The protocol is a negative selection method to scan the bacterial genome for genes which, when inactivated, cause bacteria to lose their ability to colonize the host. Mutants have a random gene inactivated by transposon-mediated insertion of a signature-tagged cassette. The signature tags are from a pool of $2 \times 10^{17}$ double-stranded 40 bp oligonucleotides. If an Input pool mutant is not recognized by the probe from the Recovery pool, the gene tagged and inactivated in that mutant is expected to have a role in pathogenicity.

In our study, we generated signature-tagged mutants of *S. choleraesuis* using Holden’s protocol (Holden & Hensel, 1998). The mutants were generated from a nalidixic acid resistant colony we derived from a virulent isolate of *S. choleraesuis* (isolate #537) supplied by Dr. Minion of Iowa State University. We established that doses of $10^7$ to $10^8$ colony forming units (cfu) induced infection in growing pigs without causing moribund pigs within four days. For protocol validation, we inoculated four 7-week-old pigs with $10^8$ cfu of a pool of 45 signature-tagged mutants of *S. choleraesuis*. Two pigs were inoculated orally and two pigs were inoculated intraperitoneally. Four days after inoculation, the pigs were euthanized and *S. choleraesuis* was isolated from the mesenteric lymph nodes of each infected pig using brilliant green agar supplemented with nalidixic acid. Total genomic DNA (chromosomal and large plasmid) was isolated from the Input pool (inoculum) and the pools of *S. choleraesuis* recovered from the inoculated pigs. The bacterial DNA from the two pigs inoculated orally were combined, whereas the DNA from the pigs inoculated intraperitoneally were left as separate samples for analysis of signature tags. Using the DNA from the three Recovery pools, radioactive probes complementary to the signature tags were synthesized by PCR. The probes were hybridized to colony blots of the inoculum to identify those mutants that were recovered from the inoculated pigs. DNA of the Input pool was run in parallel as a control.

Mutants not identified in the pool of bacteria recovered from inoculated pigs (Recovery pool) were candidates for being attenuated (having a virulence gene tagged and inactivated). Attenuation was confirmed using competitive indices after mixed challenge growth (Darwin & Miller, 1999). These indices were the ratios of mutant to wild type bacteria following expansion in mixed cultures (in vitro index) or following mixed challenge infection of pigs (in vivo index). Indices were determined for the three candidate attenuated mutants (those not in Recovery pool) and, for a control, two random non candidates (mutants in the Recovery pool). For the in vitro competitive growth, 1 ml LB broth of the mutant and of wild type *S. choleraesuis* (each approximately $10^5$ cfu/ml) were mixed and incubated until expanded more than 10,000-fold.

For the in vivo competitive growth, two pigs for each mutant were inoculated orally with $10^7$ cfu of *S. choleraesuis*; the inocula were a 1:1 mixture of mutant and wild type bacteria. Three days following inoculation, the pigs were euthanized and *S. choleraesuis* was cultured from the mesenteric lymph nodes and from pooled intestinal contents and mucosa at the ileocecal junction. The cultures were done on selective brilliant green agar supplemented with nalidixic acid. In addition, the agar used for the intestinal cultures contained streptomycin and sulfadiazine to eliminate growth of the enteric flora. After mixed expansion, either in vitro or in vivo, the phenotype of approximately 1,000 colonies was determined by replica printing the colonies to LB agar supplemented with kanamycin. (The mutants are kanamycin resistant but the wild type is sensitive and thus does not grow on the replicas.) If over 1,000 colonies were phenotyped as wild type without identifying a single mutant, the index was listed as less than 1/total number of colonies phenotyped. The in vivo competitive indices were averaged for the two pigs. The competitive index for each mutant was normalized for any differences in input ratios. The cfu in the input (the starting cultures or pig inocula) were estimated based on optical density at 600 nm and then measured by colony counts after overnight incubation on agar. The equation for the competitive index was
Figure 2. Colony hybridization of a pool of 45 signature-tagged mutants of *S. choleraesuis* with probes from the Input pool (A) and Recovery pools (B,C,D). The probes were \(^{32}\text{P}\) dCTP-labeled signature tags amplified by PCR from total DNA template from the inoculum bacteria (Input pool A) and bacteria recovered from the mesenteric lymph node 4 days after inoculating pigs orally (B: bacteria pooled from two pigs) or intraperitoneally (C, D). The large circles (mutants A5 and F1) indicate mutants with candidate pathogenicity genes tagged and therefore inactivated (attenuated mutants); these mutants were detected in the Input (A) but not Recovery pools (B,C,D). The probe for a third mutant (Figure 2, mutant B2 with small circle) failed to hybridize in one of three preparations (Figure 2, Blot C), identifying a third potential attenuated mutant.

Results

Following oral inoculation of pigs with the pool of 45 signature tagged mutants, over 10,000 cfu of *S. choleraesuis* were isolated from the mesenteric lymph nodes of each of the pigs inoculated orally (two pigs) or intraperitoneally (two pigs). The amplified labeled signature tags from these Recovery pools failed to hybridize with two mutants (A5 and F1) in colony blot hybridization assays of the inoculum (Figure 2, mutants with large circles; Input pool [Blot A] and Recovery pools [Blots B, C, and D]). Therefore, apparently these two mutants were unable to establish infection in the pigs and were candidate attenuated mutants. The probe for a third mutant (Figure 2, mutant B2 with small circle) failed to hybridize in one of three preparations (Figure 2, Blot C), identifying a third potential attenuated mutant.

(Mo/Wo)/(Mi/Wi) where *M* is mutant, *W* is wild type, *o* is cfu in output, and *i* is cfu in input. An index of 1 indicates no advantage to either the mutant or wild type isolate; mutants with an index of < 0.4 were considered attenuated (Darwin & Miller, 1999).

The first step in characterizing the candidate attenuated genes was to clone and sequence a segment of the tagged gene. Southern blot analyses were done with a \(^{32}\text{P}\) labeled kanamycin gene probe to identify restriction enzymes that excise the kanamycin cassette with a large (3 to 5 kb) segment of the adjacent genome of the mutant. *Kpn*1, *EcoRI*, or both generated large fragments in the three mutants and were used for cloning. The fragments were cloned by ligating the digested DNA from mutants into pUC18, transforming the ligated plasmid into *E. coli* DH5α, and selecting for ampicillin resistance (pUC18 encoded) and kanamycin resistance (encoded in kanamycin, signature tag cassette of the mutant). Genetic Engineering Center of the University of Illinois sequenced about 500 bp of the 5' end of the cloned *Kpn*1 segment of the first mutant. The sequencing primer was complementary to the 3' end of the kanamycin cassette (*Kpn*1 incision site is in the 5' end of the cassette) (Holden & Hensel, 1998).
To confirm attenuation of these three attenuation candidates, competitive indices were determined in vitro and in vivo. Results in Table 1 indicate that all three candidate mutants were attenuated in pigs even though two of the three (A5 and B2) may have had an advantage in culture broth (indices over 1). The competitive indices of the two negative controls (not candidates for attenuation; C4 and G3) approach 1.0 which is consistent with their presence in the Recovery pool.

Table 1. Competitive indices of five mutants from a pool of forty-five mutants screened for colonization in pigs.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Pool(^1)</th>
<th>Recovery</th>
<th>In vitro</th>
<th>Lymph node</th>
<th>Intestine</th>
<th>Attenuation in pigs(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5</td>
<td>--</td>
<td>1.6245</td>
<td>0.0016</td>
<td>0.0010</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>--</td>
<td>3.6875</td>
<td>0.0372</td>
<td>0.1521</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>--</td>
<td>0.4450</td>
<td>0.0010</td>
<td>0.0015</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>C4</td>
<td>+</td>
<td>0.4825</td>
<td>0.8278</td>
<td>0.9043</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>G3</td>
<td>+</td>
<td>0.6851</td>
<td>0.5761</td>
<td>0.5239</td>
<td>--</td>
<td></td>
</tr>
</tbody>
</table>

1 Mutants were either recognized (+) or not (–) by hybridization in colony blot assays of the Input pool probing with a \(^{32}\)P-labeled, PCR amplicon of the signature tags made from the DNA of \(S.\) *choleraesuis* in Recovery pools from inoculated pigs.

2 Indices were the ratios of mutant to wild type bacteria after mixed challenge growth that started with an inoculum of a 1:1 mixture of mutant and wild type bacteria in broth (in vitro) or in pigs (in vivo; oral inoculation with *Salmonella* culture isolation 3 days later).

3 If the in vivo index was less than \(< 0.4\), the mutant is attenuated (+); otherwise it is not attenuated (--) (Darwin & Miller, 1999).

A 3 to 8 kb segment of the tagged gene in the three attenuated mutants has been cloned into pUC18. The 5' end of one clone (mutant A5) has been sequenced, and the GenBank searched for homologues. The sequence is complementary with *hilA* of *S. typhimurium* and *S. typhi*, a positive transcriptional regulatory gene that controls expression of invasion genes (Bajaj et al., 1995, 1996).

**Discussion**

Our signature tag mutagenesis protocol is validated by the identification of candidate attenuated mutants from the test pool of 45 random mutants and the confirmation of attenuation by competitive indices from mixed challenge growth. Interestingly, mutant B2 was the least attenuated (higher competitive index than the other two attenuated mutants) and it was the only one of the three attenuated mutants that was not absent in all Recovery pools. More mutants will need to be tested to confirm if this correlation holds and how sensitive competitive indices are for the level of attenuation.

The functional genomic investigative protocol is also supported by the identification of the first gene associated with attenuation as *hilA*, a recognized virulence gene in mice studies. However, in
contrast to *S. typhimurium* in mice where only enteric infection is attenuated with mutation (Penheiter et al., 1997), *hilA* appears to have roles in both enteric and systemic invasion in pigs (the mutant failed to colonize pigs inoculated orally or intraperitoneally). Recent investigations of *hilA* function in the mouse also suggests a more complex role than previously suspected (Murray & Lee, 2000).

Our functional genomics protocol investigates virulence factors in the natural host, avoiding the problems of transferring mechanistic data between host species which can be incorrect because of host specific differences (Tsolis et al., 1999). Additional attenuated mutants will be isolated to identify other genes involved in *S. choleraesuis* virulence; some will be genes associated with virulence in other systems, some will be previously identified genes not known to have roles in pathogenesis, and others will be novel genes without previous characterization. We are extending our investigations to *S. typhimurium*. The ultimate goal is to investigate the mechanisms of *Salmonella* virulence genes and products and their functions in swine infections. These mechanisms are targets for directed, molecular methods (such as DNA vaccines) to control salmonella in pigs.

References


