Identification of Novel Genes and Pathways Affecting *Salmonella* Type III Secretion System 1 Using a Contact-Dependent Hemolysis Assay

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Type III secretion systems (T3SSs) are found in many gram-negative bacteria and are used to secrete and translocate a range of bacterial proteins known as effectors directly into the host cell (reviewed in reference 6). *Salmonella* possesses two T3SSs which are used at distinct stages of the infection process. T3SS-1, encoded within *Salmonella* pathogenicity island 1 (SPI-1), is required for the initial invasion of host cells and the induction of enteropathogenic responses (reviewed in 14, 22), while T3SS-2, encoded within SPI-2, is used in the later stages of infection, promoting survival and replication of *Salmonella* within host cells (reviewed in reference 1).

Lysis of erythrocytes in a manner dependent upon T3SSs has been observed in a number of bacteria including *Shigella*, enteropathogenic *Escherichia coli*, *Yersinia*, *Bordetella* and *Salmonella* (8, 10, 12, 15, 23). In *Salmonella*, hemolysis is dependent upon the ability of the T3SS-1 translocator proteins SipB, SipC, and SipD to form a pore in the erythrocyte membrane, causing hemoglobin leakage (15). In vitro, contact-dependent hemolysis can be monitored by incubating *Salmonella* strains with sheep red blood cells (SRBC) and measuring the absorbance at 550 nm (OD550) of the supernatant from each well (positive control), where the wild type was centrifuged at 5,700 × g for 5 min at 4°C to pellet RBC. The RBC were washed four times in phosphate-buffered saline (PBS) and then resuspended in an equal volume of PBS to give a 50% (vol/vol) SRBC-PBS suspension. Fifty microliters of bacterial culture was mixed with 50 μl of SRBC-PBS suspension in a 96-well plate and centrifuged at 1,279 × g for 10 min to aid contact between bacteria and SRBC. Following a 4-h incubation at 37°C, cells were resuspended by the addition of 150 μl of PBS and centrifuged at 1,279 × g for 10 min. A total of 100 μl of supernatant from each well was transferred to a flat-bottomed 96-well plate, and the absorbance was recorded at 550 nm to detect the presence of released hemoglobin.

From this initial screen of 5,700 *Salmonella enterica* serovar Typhimurium mutants for defects in type III secretion system 1 (T3SS-1)-mediated contact-dependent hemolysis to identify novel genes and pathways affecting the activity of T3SS-1. Our data suggest that previously unrecognized factors such as type I fimbriae may modulate the expression, activity, or deployment of this key virulence factor.

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less hemolytic than those in late exponential phase. Though hemolytic activity was sensitive to growth phase, it was not affected by the starting bacterial density as diluting cultures through 80, 60, 40, and 20% of the starting OD$_{550}$ did not affect hemolytic activity. Thus, if equivalent numbers of bacteria enter the assay and if mutants with impaired growth are rejected, one may anticipate that mutants with altered hemolytic activity carry mutations that directly affect the expression, function, or deployment of T3SS-1.

**Identification of mutants with reduced T3SS-1-mediated hemolytic activity.** For the remainder of this study we focused on mutants that we had classified as reduced or negative in the hemolysis screen. Since a large number of mutants exhibiting impaired hemolytic activity were predicted to have defects in SPI-1, the chromosomal DNA region encoding T3SS-1, a PCR screen was performed to identify and eliminate such mutants. Four primers spanning the DNA region encoding T3SS-1, a PCR screen was performed to identify which mutated genes were in operons and therefore potentially sensitive to polar effects. Of those genes identified as potentially sensitive to polar effects, one may anticipate that mutants with altered hemolytic activity carry mutations that directly affect the expression, function, or deployment of T3SS-1.

<table>
<thead>
<tr>
<th>Unique mutant identifier</th>
<th>Gene (synonym)</th>
<th>Function</th>
<th>Hemolytic activity (% of wild type ± SD) in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>STM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P22 transductant</td>
</tr>
<tr>
<td>M7f4</td>
<td>hns</td>
<td>DNA binding protein, global regulator</td>
<td>13.0 ± 4.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>M17c10</td>
<td>stpA</td>
<td>HNS-like global regulator</td>
<td>39.7 ± 26.6</td>
</tr>
<tr>
<td>M28f10, M45f10, M49d10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>sirA (uvrY)</td>
<td>Response regulator controlling virulence gene expression</td>
<td>5.2 ± 6.7</td>
</tr>
<tr>
<td>M60f1</td>
<td>rfaL</td>
<td>LPS core biosynthesis; O-antigen ligase</td>
<td>23.9 ± 4.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M7a4</td>
<td>rfaG</td>
<td>LPS core biosynthesis; glucosyltransferase I</td>
<td>47.9 ± 5.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M37f12</td>
<td>rfaI</td>
<td>LPS core biosynthesis; (glycosyl)lipopolysaccharide-alpha-1,3-galactosyltransferase</td>
<td>3.6 ± 5.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M5b4</td>
<td>rfbU</td>
<td>LPS O-antigen biosynthesis</td>
<td>34.7 ± 6.9</td>
</tr>
<tr>
<td>M31f1</td>
<td>rfbI</td>
<td>LPS O-antigen biosynthesis</td>
<td>46.9 ± 7.8</td>
</tr>
<tr>
<td>M33d10, M38d10</td>
<td>rfbF</td>
<td>LPS O-antigen biosynthesis</td>
<td>30.1 ± 16.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M31g3</td>
<td>cpsB (rfbM, manC)</td>
<td>Mannose-1-phosphate guanylyltransferase, involved in colanic acid biosynthesis</td>
<td>41.4 ± 24.4</td>
</tr>
<tr>
<td>M14h4</td>
<td>spr (yeiV)</td>
<td>Putative lipoprotein, suppresses thermosensitivity of pcv mutants at low osmolality</td>
<td>56.3 ± 17.6</td>
</tr>
<tr>
<td>M16f3</td>
<td>fimW</td>
<td>Regulator of type I fimbrae expression</td>
<td>42.9 ± 4.9</td>
</tr>
<tr>
<td>M12d5, M14g3, M14h5, M13e9, M37e9</td>
<td>fadD</td>
<td>Long chain fatty acid acyl-CoA synthetase</td>
<td>16.8 ± 22.6</td>
</tr>
<tr>
<td>M5c65</td>
<td>ackA</td>
<td>Acetate kinase A</td>
<td>48.0 ± 14.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>M49g1</td>
<td>emcD</td>
<td>3-Dehydroquinate dehydrogenase</td>
<td>45.2 ± 13.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M5e412</td>
<td>deuD</td>
<td>ATP-independent RNA helicase</td>
<td>67.2 ± 22.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>M34c4</td>
<td>uvrB</td>
<td>Part of ATP-dependent DNA excision repair enzyme complex</td>
<td>41.6 ± 20.8</td>
</tr>
<tr>
<td>M41a5, M47a5, M54a5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>agaR</td>
<td>Transcriptional repressor of aga operon</td>
<td>53.0 ± 14.3</td>
</tr>
<tr>
<td>M46g10</td>
<td>emcV (yibP)</td>
<td>Peptidoglycan hydrolase; required for cell division</td>
<td>39.1 ± 6.6</td>
</tr>
<tr>
<td>M53b8</td>
<td>yqjB (ecfM)</td>
<td>Unknown</td>
<td>55.3 ± 12.8</td>
</tr>
<tr>
<td>M44d6, M14h10</td>
<td>yfgA/yfgB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Unknown</td>
<td>69.8 ± 15.5&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>M50d1</td>
<td>ycgL/minC&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Unknown (ycgL); cell division inhibitor (minC)</td>
<td>61.0 ± 27.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> Transposition insertion between the two genes.

<sup>b</sup> Results obtained from additional assay with bacteria corrected to the same OD$_{550}$.

<sup>c</sup> STM mutant used for P22 transduction.

<sup>d</sup> Defined deletion mutant was used rather than a P22 transductant.

This method eliminated approximately 50% of the mutants. The remaining mutants were then identified by subcloning and sequencing to map transposon insertion sites as previously described (17) with the exception that sequencing was carried out in-house using Beckman CEQ8000 sequencers.

Twenty-three genes outside of SPI-1 were identified and are listed in Table 1. Some genes were previously known to affect T3SS-1 activity, for example, sirA encoding a global response regulator (11). This provided further evidence that the hemolysis assay was a suitable method for identifying genes and pathways affecting T3SS-1. Genes not previously known to affect *Salmonella* T3SS-1 were involved in a number of different biochemical pathways including lipopolysaccharide (LPS) biosynthesis, fimbriae expression, and DNA repair. Multiple independent mutations in some systems were identified; for example, six separate genes in the LPS biosynthetic pathway were identified, confirming the importance of this pathway in T3SS-1-mediated hemolysis and markedly reducing the likelihood that the observed phenotypes are due to second-site defects. Further, the assay reproducibly detected the involvement of specific genes; for example, *fadD* mutants were isolated five times, and *sirA* mutants were isolated three times.

The serovar Typhimurium LT2 genome was analyzed to identify which mutated genes were in operons and therefore potentially sensitive to polar effects. Of those genes identified as being located in an operon and therefore having potential polar effects, the majority were in LPS genes. The involvement...
of LPS per se in regulation of T3SS-1 activity should be regarded with caution as it is required for membrane integrity and proper insertion and folding of membrane proteins. Indeed, some lipid A mutations have already been shown to impair T3SS-1 function (24).

To confirm that the phenotype of reduced T3SS-1-mediated hemolysis was due to mutation of the gene identified by transposon insertion sequencing and not to secondary defects, P22 transduction of a subset of the mutants into the archived serovar Typhimurium 4/74 parent strain was performed. Mutation of the desired gene in the resulting transductants was confirmed by PCR with gene-specific primers. The P22 transductants were restested three times in the hemolysis assay and displayed a similar phenotype as the original STM mutant (Table 1), confirming that the effect on T3SS-1-dependent hemolysis was most likely due to disruption of the gene identified by sequencing of the transposon insertion.

For further validation of the screen, fadD was selected as this gene had previously been identified in a screen of mutants with reduced expression of the T3SS-1 transcriptional regulator hilA (13). fadD encodes the long-chain fatty acyl-CoA synthetase, which is associated with the inner bacterial membrane and catalyzes the esterification of long-chain fatty acids into fatty acyl-CoA molecules (reviewed in reference 4). The fatty acyl-CoA molecules can be incorporated into phospholipids for membranes or can be further degraded via the beta-oxidation pathway. A defined fadD deletion mutant was constructed using overlapping PCR followed by allelic exchange with the pDM4 suicide vector (16). The first 300 and last 325 nucleotides of fadD were amplified by PCR using the primer pair FadD_5'/H11032 (5'-CTCCCTCAGATGGAAAGGT TTGGCTT-3') and FadD_int2 (5'-CAGAATCTATCTTCTTCTAATCATGCCAGCGGTAAAATACC-3') and the pair FadD_3'xba (5'-CTCTCTCAGATGGAAAGGT TTGGCTT-3') and FadD_int3 (5'-TTACGCGCTGGCATGATTAAGA AACGATTACGATTGCTCTGATACGAC-3'). The resulting mutant, serovar Typhimurium 4/74 Nalr ΔfadD contained just the start and stop codons of the fadD gene. For use as a comparison in some assays a fimA deletion mutant lacking the fimbral subunit (serovar Typhimurium 4/74 Nalr ΔfimA) was also created using the same method with the primer pair fimA_xho_5' (5'-CTCCTCGAGATGGAAAGGT TTGGCTT-3') and fimA_mut_3' (5'-GCCGTTCCTGCCGAAGTGACCTC-3') and the pair fimA_xba_3' (5'-CTCTCTCAGATGGAAAGGT TTGGCTT-3') and fimA_mut_5' (5'-TGTAATTACGAGGAAATCTCAAGGAGGAATCTCATGATGGAAAGGT TTGGCTT-3').

The expected phenotypes of the mutants were confirmed by blotting and mannose-sensitive yeast agglutination. For the dot blot, bacterial strains were cultured statically in 10 ml of LB medium containing nalidixic acid (20 μg/ml) at 37°C for 48 h. Whole-cell lysates were prepared by centrifuging 10 ml of culture, washing the bacterial pellet in PBS, and resuspending in 50 μl of PBS. The lysates were normalized to equivalent bacterial numbers, serial dilutions were made, and samples were heated at 95 to 100°C for 8 min. Four-microliter spots were applied to Hybond ECL membrane (GE Healthcare) in two applications of 2 μl. The membrane was blocked in 3% nonfat dried milk in PBS, and immunoblotting was performed using anti-FimA-glutathione S-transferase antisera (1:100), a kind gift from Andreas Ba¨umler, and goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (1:10,000; Sigma). Type I fimbriae were detected in the serovar Typhimurium 4/74 Nalr wild-type sample at a dilution of up to 1:200 (Fig. 1). In contrast, in the serovar Typhimurium 4/74 Nalr ΔfimA mutant, type I fimbriae were still detected at the highest dilution of 1:500, indicating that this strain produces an elevated level of FimA and, presumably therefore, more type I fimbriae than the wild-type strain. FimA was not detected in the serovar Typhimurium 4/74 Nalr ΔfimA strain.

As the dot blot assay used whole-cell lysates and could not distinguish surface-expressed type I fimbriae, a yeast cell slide agglutination assay was performed based on an adaptation of a method by Roe et al. (19). Briefly, bacterial strains were cultured overnight statically at 21°C and then diluted 1:2 into 10 ml of fresh LB medium and cultured statically for 90 min at 37°C. These culture conditions mimicked those of the hemo-
Typhimurium 4/74 Nalr played agglutination only after a 20-min incubation. The sero-
of 3% (wt/vol) D-mannose abolished agglutination in all cases, i fimbriae (19), did not cause yeast cell agglutination. Addition 
murium 4/74 Nalr indicating that yeast cell agglutination in the serovar Typhi-
rhagic

lysis assay, and in addition to providing data on surface expres-
sion levels of type I fimbriae, the assay provided data that could be 
correlated to type I fimbrial expression patterns in the 
hemolysis assay. Bacteria were pelleted by centrifugation and 
resuspended in PBS, with the OD_{600} of the cultures adjusted to 
1.75. Agglutination was assayed on glass slides mixing 15 μl of 
bacteria with an equal volume of 10 mg/ml *Saccharomyces cerevisiae* cell suspension (Sigma). Slides were incubated at 4°C and 
monitored periodically for agglutination. Mannose inhibitor-
ning of agglutination was confirmed using 3% (wt/vol) D-manne-
ose in the yeast suspension. Serovar Typhimurium 4/74 Nal' ΔfimW' displayed strong yeast cell agglutination within 10 min, whereas serovar Typhimurium 4/74 Nal' wild-type bacteria dis-
played agglutination only after a 20-min incubation. The sero-
var Typhimurium 4/74 Nal' ΔfimA mutant, as expected, did not 
agglutinate yeast cells. A negative control of enterohemor-
rhagic *E. coli* O157:H7 (TUV930), which does not express type I 
fimbriae (19), did not cause yeast cell agglutination. Addition of 
3% (wt/vol) D-mannose abolished agglutination in all cases, 
indicating that yeast cell agglutination in the serovar Typhi-
murium 4/74 Nal' ΔfimW mutant and parent strain was due to 
surface expression of type I fimbriae.

In the hemolysis assay the defined fimW mutant induced less 
lysis (25.3% ± 11.5%) of SRBC than serovar Typhimurium 
4/74 Nal' wild type (Table 1). Based on these data, we hypothe-
sized that a possible mechanism of action for the role of type 
I fimbriae in T3SS-1 activity is a spatial interference effect, 
whereby the increased amount of type I fimbriae on the surface of 
the fimW mutant prevents the T3SS-1 from efficiently con-
tacting eukaryotic cells. Consistent with this hypothesis, re-
moval of type I fimbriae via mutation of fimA resulted in 
increased hemolysis (113.2% ± 23.0%) compared to the wild-
type strain.

The *fadD* and *fimW* mutants behaved differently from wild-
type bacteria in another T3SS-1-dependent assay. A second 
read-out of T3SS-1 function, the gentamicin protection assay, 
was carried out to measure bacterial invasion of cultured epithe-
rial cells since invasion is a major function of *Salmonella* 
T3SS-1.

Bacterial strains were cultured statically overnight at 25°C in 
10 ml of LB medium containing appropriate antibiotics and 
then diluted 1:2 in 10 ml of fresh LB medium and cultured 
statically for 90 min at 37°C. Confluent monolayers of HeLa 
cells in 24-well plates were washed in PBS and incubated for 30 
min at 37°C in 5% CO_{2} in fresh Dulbecco's modified Eagle's 
medium containing 10% (vol/vol) fetal calf serum. Approxi-
ately 5 × 10^{6} bacteria were added to each well of HeLa cells 
(multiplicity of infection of 2.5), and plates were centrifuged 
to facilitate contact of the bacteria with the HeLa cells. Following 
a 1-h infection at 37°C in 5% CO_{2}, cells were washed three 
times in PBS and fresh Dulbecco's modified Eagle's medium 
containing 10% fetal calf serum, and 150 μg/ml gentamicin was 
added. Cells were incubated for 1 h more and then were 
washed three times in PBS and lysed by the addition of 100 μl 
of 1% Triton X-100 in PBS for 5 min. PBS (900 μl) was added 
to each well, and bacteria were plated onto L-agar plates con-
taining nalidixic acid (20 μg/ml) for enumeration of invaded 
bacteria. Each bacterial strain was assayed in triplicate in the 
invasion assay.

*Salmonella* serovar Typhimurium 4/74 Nal' ΔfadD_{300-1360} 
showed an 80% decrease in invasion compared to serovar 
Typhimurium 4/74 Nal' wild-type (Fig. 2a), and a control of 
serovar Typhimurium 4/74 harboring a transposon insertion in 
a gene encoding a structural component of T3SS-1, *prgH* 
(*prgH*-miniTn5Km2) (17), was unable to invade HeLa cells,
indicating that the assay was dependent on a functional T3SS-1. Under these conditions, serovar Typhimurium 4/74 \( \Delta \text{fimW} \) was more invasive than the wild-type strain (Fig. 2a), contrasting with data from the hemolysis assay. However, when the invasion assay was repeated in the presence of 0.2 M D-mannose to block secondary effects of the FimH adhesin, serovar Typhimurium 4/74 \( \Delta \text{fimW} \) showed a 25% decrease in invasion compared to the wild-type strain (Fig. 2b). The FimH adhesin of type I fimbriae binds mannosylated glycoproteins on or secreted by eukaryotic cells, facilitating adhesion of bacteria to eukaryotic cells; this observation suggests that in the initial invasion assay (Fig. 2a), results were biased by increased type I fimbriae on the \( \text{fimW} \) mutant strain, thereby resulting in increased adherence to the HeLa cells. This is supported by reports that \textit{Salmonella} type I fimbriae mediate adhesion in vitro to a variety of cell types (3, 7, 20) and contribute to colonization of pigs in vivo (2). The differences in the ability of the \( \text{fadD} \) and \( \text{fimW} \) mutants to induce T3SS-1-mediated contact-dependent hemolysis may be partially due to differences in T3SS-1 secretion. Protein secretion by \textit{Salmonella} T3SS-1 can be induced in vitro by a temperature shift of the bacterial culture from 25°C to 37°C, resulting in the secretion of large quantities of T3SS-1 effector proteins into the culture supernatant (2b). We used a modification of this method to mirror the culture conditions used in the hemolysis assay. Briefly, bacterial strains were cultured statically overnight in 10 ml of LB medium at 25°C, diluted 1:2 into 10 ml of fresh LB medium, and cultured statically for 90 min at 37°C. Ten milliliters of culture was pelleted by centrifugation (10,000 \( \times \) g for 10 min) and resuspended in 7.5 ml of fresh LB medium to remove the background of secreted proteins. Five milliliters was added to 5 ml of PBS and incubated statically for 4 h at 37°C. At the end of the incubation period, bacteria were pelleted by centrifugation, and the supernatant was passed through a 0.45-\( \mu \)m-pore-size low-protein-binding filter. Secreted proteins were collected by incubating the filtered supernatant with 30 \( \mu \)l of StrataClean resin (Stratagene). The resin was washed in PBS and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Proteins were separated by SDS-PAGE and stained with Coomassie blue. Western blotting of secreted proteins was performed using Hybrid-ECL membrane, blocked in 3% (wt/vol) nonfat dried milk and incubated with anti-SipC (clone F569-c6c6) or anti-SipB (clone F575-AB4) monoclonal antibodies (generated in-house) at 1 \( \mu \)g/ml (diluted in 1% [wt/vol] bovine serum albumin in Tris-buffered saline), followed by donkey anti-mouse horseradish peroxidase-conjugated secondary antibody (GE Healthcare) at a concentration of 1:10,000. Blots were developed using chemiluminescence (ECL kit, GE Healthcare).

Secreted protein profiles from both serovar Typhimurium 4/74 \( \Delta \text{fimW} \) and serovar Typhimurium 4/74 \( \Delta \text{fadD}_{\text{COD,1350}} \) showed reduced amounts of T3SS-1-secreted proteins (Fig. 3a), with the \( \text{fadD} \) mutant showing the least amount of secreted proteins. Western blotting confirmed that both the \( \text{fadD} \) mutant and the \( \text{fimW} \) mutant secreted less SipB and SipC T3SS-1 translocator proteins than the wild-type strain (Fig. 3b). Samples of the bacterial cultures were taken at 0 and 4 h postinoculation for viable counts. Bacterial numbers were similar for all cultures at each time point; thus, the mutations did not affect bacterial growth (data not shown).

**FIG. 3.** The \( \text{fimW} \) and \( \text{fadD} \) mutants secrete smaller amounts of T3SS-1-secreted proteins than wild-type bacteria. Bacteria were grown statically overnight at 21°C, subcultured 1:2 into 20 ml, and cultured statically at 37°C for 90 min. Ten milliliters of culture was pelleted by centrifugation and resuspended in 7.5 ml of fresh LB medium, and 5 ml was added to 5 ml of PBS and incubated statically for 4 h at 37°C. Secreted proteins were isolated from culture supernatants and subjected to SDS-PAGE followed by Coomassie staining (a) or Western blotting with anti-SipC and anti-SipB antibodies (b). WT, wild type.
this stage we are unable to entirely rule out some role for spatial interference or effects on membrane integrity. Indeed, it is likely that other genes we identified in the hemolysis screen that are involved in bacterial surface structures such as LPS may have a spatial interference explanation for their lack of hemolysis, either impeding the ability of T3SS-1 to contact erythrocytes or affecting assembly of T3SS-1 in the bacterial membrane. The effect of LPS mutations on type III secretion has been observed in *Shigella*, where a mutant defective in LPS O-antigen glucosylation showed reduced epithelial cell invasion in vitro compared to wild-type bacteria (25). This is because glucosylation of LPS causes a conformational change in the O antigen, shortening the distance it extends from the *Shigella* surface and thereby optimizing access of the T3SS to the epithelial cells (25). In *Yersinia* increasing the length of the adhesin YadA reduced Yop effector translocation via the T3SS (18), providing further evidence of how bacterial surface structures impact on T3SS function.

Other genes identified in our screen may have more complex, and as yet unknown, explanations for their inability to induce T3SS-1-mediated contact-dependent hemolysis, and characterizing these mutants could potentially identify new targets for therapeutics.

This work has shown that the *Salmonella* T3SS-1-dependent hemolysis assay developed by Miki and colleagues (15) is a useful method for high-throughput screening of a large number of mutants for T3SS-1 activity. The method has numerous advantages including speed, reliability, and sensitivity. It should be capable of being readily adapted to other bacteria demonstrating T3SS-dependent hemolytic activity to identify novel genes and pathways affecting type III secretion in these organisms.

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