Yeast two-hybrid system survey of interactions between LEE-encoded proteins of enteropathogenic Escherichia coli

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Many Gram-negative pathogens employ a specific secretion pathway, termed type III secretion, to deliver virulence effector proteins directly to the membranes and cytosol of host eukaryotic cells. Subsequent functions of many effector proteins delivered in this manner result in subversion of host-signalling pathways to facilitate bacterial entry, survival and dissemination to neighbouring cells and tissues. Whereas the secreted components of type III secretion systems (TTSSs) from different pathogens are structurally and functionally diverse, the structural components and the secretion apparatus itself are largely conserved. TTSSs are large macromolecular assemblies built through interactions between protein components of hundreds of individual subunits. The goal of this project was to screen, using the standard yeast two-hybrid system, pair-wise interactions between components of the enteropathogenic Escherichia coli TTSS. To this end 37 of the 41 genes encoded by the LEE pathogenicity island were cloned into both yeast two-hybrid system vectors and all possible permutations of interacting protein pairs were screened for. This paper reports the identification of 22 novel interactions, including interactions between inner-membrane structural TTSS proteins; between the type III secreted translocator protein EspD and structural TTSS proteins; between established and putative chaperones and their cognate secreted proteins; and between proteins of undefined function.

INTRODUCTION

There are only a limited number of ways by which Gram-negative bacteria can transport proteins across their unique double membrane (Thanassi & Hultgren, 2000). Most secretion substrates are exported via the General Secretion Pathway (type II secretion), and are synthesized with a hydrophobic amino-terminal signal peptide. Type II secretion is sec-dependent and the two bacterial membranes are crossed in distinct steps.

In contrast, the type III secretion pathway is sec-independent and secreted proteins, which do not have cleaved signal peptides, are transported across both membranes in a single step. Type III secretion systems (TTSSs) are utilized by many Gram-negative pathogens to directly translocate virulence factors into host cells. These virulence factors are responsible for the subversion of host cell signal transduction pathways for the benefit of the bacterium and result in bacterial adhesion, invasion and disease (Hueck, 1998).

The type III secretion apparatus is a multi-component organelle assembled from the products of approximately 20 genes. Many components are broadly conserved amongst both virulence and flagellar TTSSs (Hueck, 1998). The secretion apparatus that can be isolated from bacterial membranes and visualized by transmission electron microscopy is referred to as the needle complex (NC). Assembly of the NC occurs in stages (Sukhan et al., 2001). Initially, in a sec-dependent manner, the membrane-bound components are exported to form the foundation of the NC (consisting of stacked membrane rings joined by a central rod). This is followed by assembly of the inner-membrane-associated machinery, which enables secretion of components constituting the ‘needle’ and more distal components of the apparatus, including the translocator proteins, which directly interact with the eukaryotic plasma membrane to facilitate translocation of effector proteins (Buttnner & Bonas, 2002). The completed apparatus can transport effector proteins from the bacterial cytoplasm directly to the host cell cytoplasm, crossing three membranes in a single step.

Enteropathogenic Escherichia coli (EPEC) is a Gram-negative, non-invasive enteric bacterial pathogen that relies on a TTSS to colonize the intestine through formation of distinct attaching and effacing (A/E) lesions (reviewed by...
Frankel et al., 1998), which are characterized by local destruction of brush border microvilli and rearrangement of the host cell cytoskeleton. The genes encoding the A/E phenotype are encoded on a pathogenicity island termed the locus of enterocyte effacement (LEE), which is also present in other A/E lesion-causing bacteria such as enterohemorrhagic E. coli, and Citrobacter rodentium (McDaniel et al., 1995). The complete region consists of 41 open reading frames (Elliott et al., 1998). Genes homologous to Yersinia type III secretion (ycs) genes are named E. coli secretion (esc) genes with the same suffix as the Yersinia homologue, and those without a Yersinia homologue, but with an experimentally defined role in secretion, are named secretion of E. coli protein (sep) genes. Genes encoding secreted proteins and their chaperones are termed esp (for E. coli secreted protein) and ces (for chaperone for E. coli secretion) respectively. The remaining genes encode either putative type III secretion proteins without homologues in other systems, or genes that have retained the nomenclature ascribed to their initial characterization, such as eae (intimin) (Jerse et al., 1990), or ler (LEE-encoded regulator; Mellies et al., 1999).

Over the last few years much has been learnt about individual components of the EPEC type III secretion apparatus and their roles within this structure. Only one EPEC protein, EscC, is known to be located in the outer bacterial membrane. EscC is a member of the secretin superfamily, and is believed to be secreted by the general secretion pathway to the outer membrane, where it exists as a large homomultimeric ring complex as has been observed for other members of the secretin family (Koster et al., 1997; Crago & Koronakis, 1998; Nouwen et al., 2000). These complexes permit the passage of exported substrates across the outer membrane. Based on homology with the Shigella system, part of the EscC ring is thought to project into the periplasm, where it can interact with inner-membrane proteins to form the central rod observed by electron microscopy (Blocker et al., 2001; Schuch & Maurelli, 2001). The conserved inner-membrane protein EscJ is similarly believed to extend into the periplasm. Esc homologues share sequence similarity with a domain of the flagellar protein FliF, which polymerizes to form the inner-membrane MS rings of the flagellar basal body (Suzuki et al., 1998). Other conserved TTSS proteins, including EscR, S, T, U and V, are predicted to span the inner membrane, although their precise function and localization within the apparatus is unclear. The EPEC TTSS also has several cytoplasmic components, including EscN, a highly conserved ATPase, the LEE-encoded regulator (Ler) and several chaperones.

The needle structure of the EPEC TTSS is composed of EscF. Only EscF has been shown to be required for assembly of the needle (Wilson et al., 2001), and an escF mutant is unable to secrete additional TTSS substrates, indicating that the needle must be formed prior to secretion of other more distal components. The TTSS of EPEC has a unique 12 nm diameter filamentous structure extending from the end of the EscF needle (Knutton et al., 1998; Daniell et al., 2001), which forms a direct link between the bacterium and the host cell (Knutton et al., 1998). Evidence suggests that the secreted protein EspA is the major component of this filamentous structure (Knutton et al., 1998; Sekiya et al., 2001; Daniell et al., 2001), although the translocator protein EspD, which is presumed to be exported through the filament, is also required for filament assembly (Kresse et al., 1999). EspD is similar to the Yersinia YopB and Shigella IpaB proteins, which, in complex with a second translocator (EspB, YopD and IpaC, respectively), are reported to form a pore in the host cell membrane that facilitates subsequent entry of additional virulence proteins (Buttner & Bonas, 2002).

TTSS substrates are recognized to have a presecretory requirement for the function of small chaperone molecules. Within the LEE regions CesT has been identified as a cytosolic chaperone with dual specificity assisting in translocation of both the translocated intimin receptor (Tir; Abe et al., 1999; Elliott et al., 1999) and Map (Creasey et al., 2003), and CesF was shown to chaperone EspF (Elliott et al., 2002). In contrast, maximal secretion of EspD requires the function of two chaperones, CesD and CesD2, which have been localized to the inner membrane (Wainwright & Kaper, 1998; Neves et al., 2003), CesD is also required for maximal secretion of EspB (Wainwright & Kaper, 1998). As yet, no chaperones have been reported for EspA or the effector EspG.

The exact structure of the TTSS apparatus remains elusive despite much work in the field. Given the complex and numerous protein interactions that must occur between individual components in both the construction and function of the TTSS, an investigation of individual protein interactions is a good first approach upon which to build an initial picture of the overall structure. Recent studies have made effective use of the yeast two-hybrid system (Y2HS) to identify and investigate pair-wise protein interactions. This system has been used widely in the study of TTSSs to investigate binding partners of individual proteins (Elliott et al., 1999; de Grado et al., 1999; Daniell et al., 2001; Schuch & Maurelli, 2001), targets of effector proteins within the host cell (Hartland et al., 1999; Kodama et al., 2002) and more global studies of the entire TTSS (Minamino & Macnab, 2000a; Jackson & Plano, 2000; Page et al., 2001, 2002). However, no comprehensive Y2HS screen of an entire TTSS has been reported so far. The aim of this project was to use a global Y2HS screen to identify novel pair-wise protein interactions involved in the assembly and function of the EPEC TTSS. Here we present our findings and discuss the usefulness of this system for studying TTSSs.

**METHODS**

**Strains and plasmids.** The plasmids used in this study are listed in Table 1. Bacterial strains EPEC E2348/69 (wild-type EPEC O127:H6 isolated from an outbreak in Taunton, UK; Levine et al.,
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Yeast two-hybrid system. The LEE-encoded genes were amplified from EPEC E2348/69 genomic DNA and cloned into the EcoRI/BamHI, EcoRI/SalI or BamHI sites of yeast two-hybrid vectors pGBT9 and pGAD424, creating fusions to the binding and activation domains of the yeast transcriptional activator GAL4 respectively. Both vectors carry an Ap<sup>+</sup> gene for selection in bacterial hosts; in addition, pGBT9 carries the yeast TRP1 marker gene and pGAD424 carries the yeast LEU2 marker gene. Each pGBT9 ‘bait’ construct was introduced singly into S. cerevisiae strain Pf69-4A using the high-efficiency lithium acetate transformation procedure described by Geitz & Schiestl (1995) and selected on medium lacking Trp. The pGAD424 ‘prey’ constructs were divided into eight pools (Table 2); each of the yeast strains harbouring a bait construct was transformed with the eight pools of prey plasmids and selected on medium lacking Trp and Leu. Pf69-4A contains three separate reporter genes (HIS3, ADE2 and lacZ) each under the independent control of three different GAL4 promoters (GAL1, GAL2 and GAL7, respectively).

Table 2. Pools of prey plasmids used in the screening

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*Unstable in the yeast vectors, therefore uncloned.
that provide a high level of sensitivity with respect to detecting weak interactions, coupled with a low background of false positives (James et al., 1996). Co-transformants were replica-plated onto medium lacking Trp, Leu, Ade and His to select for activation of the ADE2 and HIS3 reporter genes. When a bait protein was found to interact with a pool of prey proteins, the individual prey constructs making up that pool were introduced into the yeast strain harbouring the bait construct to identify the interacting protein(s).

Activation of the lacZ reporter gene in yeast strains harbouring single constructs or a pair of interacting constructs was assessed by quantification of β-galactosidase activity in cell extracts according to the liquid assay method of Miller (1972) using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate.

**Construction of EspD truncations.** Truncations of espD lacking either of the hydrophobic domains were constructed by inverse PCR using pICCC70 as a template. Primer pair 5’-ATATGGATCCGCA-CTCTGGGCTGTTGTTGCTATTAGT-3’/5’-ATATGGATCCCAATTTC-ACCTTTGTGCTCTTCTTGCGC-3’ was used to remove the N-terminal hydrophobic domain (residues 177-197) and primer pair 5’-ATATGGATCCGGGGGTGTTCACTTATATCTAAAGTT-3’/5’-ATATGGATCCCTTGCGCTGTTTTATAGGCTG-3’ was used to remove the C-terminal hydrophobic domain (residues 234-254).

**Structural predictions.** Signal peptides were identified using SignalP V2.0.2 (Nielsen et al., 1997). Predictions of molecular mass and pI were obtained using ProtParam tool from ExPASy (Wilkins et al., 1998). Possible membrane-spanning regions were identified using DAS (Csérapek et al., 1997), TMHMM V2.0 (Krogh et al., 1997), HMMTOP V2.0 (Tusnady & Simon, 2001), TMHMM V2.0 (Krogh et al., 2001; Sonnhammer et al., 1998) and TopPred (Claros & von Heijne, 1994).

**RESULTS**

**Cloning the LEE genes**

Primers incorporating appropriate restriction sites were designed to amplify all of the LEE-encoded genes from the prototype EPEC strain E2348/69 not previously cloned into the yeast matchmaker vectors, with the exception of ler (a DNA-binding protein and a regulator of LEE gene expression; Mellies et al., 1999) and eae (an outer-membrane protein not involved in TTSS assembly and function; Fig. 1, Table 3). Genes were amplified using high-fidelity Deep Vent DNA Polymerase (NEB) using standard thermal cycling protocols. The amplified LEE genes were cloned into both pGBT9 and pGAD424 yeast matchmaker vectors, with the exception of escV and escN, which were unstable. The physical properties of some proteins, particularly the presence of hydrophobic domains such as transmembrane segments or signal peptides, inhibit their nuclear localization within the yeast cell (Brent & Finley, 1997); therefore the mature forms of proteins with predicted sec-dependent signal peptides (rOrf1, rOrf3, EscC, Esc and Orf18) were cloned into the yeast matchmaker vectors.

**Strategy for screening LEE-encoded proteins using the Y2HS**

Our aim was to test all possible combinations of the 37 cloned genes for pair-wise protein interactions, a total of 1369 potential interactions. To reduce the number of initial transformations, our approach was to screen individual ‘baits’ (pGBT9 constructs) against a pool of ‘preys’ (pGAD424 constructs) in the yeast. Each bait plasmid was initially transformed into the host yeast strain PJ69-4A and transformants selected on plates lacking tryptophan. Eight pools, each containing five prey plasmids (Table 2), were subsequently transformed into the previously selected yeast bait strains.

Transformants were first selected on plates lacking tryptophan and leucine, then the colonies were replicated onto plates lacking tryptophan, leucine, adenine and histidine to select for protein interaction on the basis of the activation of the ADE2 and HIS3 reporter genes. When a bait plasmid was found to interact with a pool of prey plasmids, the yeast strain harbouring the bait plasmid was transformed with each member of the pool individually to identify the specific interacting partner(s). Protein interactions, indicated by growth on selective media, were further confirmed in the yeast by assessing relative expression from the lacZ reporter gene using quantitative β-galactosidase assays. Results were expressed as the fold increase in β-galactosidase activity compared to strains harbouring the bait or prey plasmids alone.

**Y2HS screening identifies multiple protein interactions**

Twenty-five pairs of interacting proteins were identified from the YTH screen and graded from strong to weak depending on whether yeast colonies were observed on selective media (positive YTH phenotype) after 2–3 days (++) or 4-7 days (+) or 7–14 days (+) (Table 4). All interactions previously identified using the Y2HS in other studies were again identified during this study (Table 4), confirming the integrity of the screening system.

In general it was observed that proteins that interact strongly
with themselves did not perform well as baits for other proteins, e.g. EscR and EspD, and some proteins were self-activators (Table 5). Altogether, 22 of the interactions are novel and have not previously been described. Most of the interacting pairs can be divided into the following three categories.

(i) Structural proteins. One clear set of interactions involved proteins known to be located in, or associated with, the inner membrane, namely EscR:EscR, EscS:EscR and EscU:EscR (Table 4). Two further interactions, EscU:rOrf8 and SepZ:EscR, involving partners predicted to be associated with the inner membrane were also obtained; however none of these interactions were observed when screening in the alternate orientation. Moreover, yeast expressing EscU:EscR and SepZ:EscR did not exhibit a detectable increase in β-galactosidase activity, within the limits of our system, indicating a failure to activate the lacZ reporter gene. One limitation of this system is that interactions are detected within a hydrophilic compartment; therefore hydrophobic domains, such as transmembrane domains, may aggregate. We cannot rule this out for these interactions; however, their validity is suggested by virtue of proximity of the proteins to each other in the physiological setting, as defined by previous studies (Hueck, 1998).

A further interaction was detected between the EscD and EscC structural proteins. EscD is predicted to span the inner membrane with a substantial part of the protein located in the periplasm, where it could interact with EscC. This is in contrast to the Shigella TTSS, where the EscC homologue (MxiD) interacts with the EscJ homologue (MxiJ; Schuch & Maurelli, 2001).

(ii) Translocator and inner-membrane components. An unexpected set of interactions was that of structural proteins EscR, EscS, EscU, SepZ and rOrf1 with the secreted protein EspD (Table 4). EspD is known to be required for polymerization of EspA filaments (Knutton 2098).
Table 4. Yeast two-hybrid results

<table>
<thead>
<tr>
<th>Bait</th>
<th>Prey</th>
<th>Growth</th>
<th>β-Galactosidase units (mean ± SD, n = 9)</th>
<th>Fold increase in β-gal activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously identified interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tir</td>
<td>CesT</td>
<td>+++</td>
<td>27.2 ± 1.4</td>
<td>15.8</td>
</tr>
<tr>
<td>CesT</td>
<td>Tir</td>
<td>+++</td>
<td>19.6 ± 0.2</td>
<td>34.8</td>
</tr>
<tr>
<td>CesT</td>
<td>CesT</td>
<td>+++</td>
<td>10.8 ± 1.0</td>
<td>21.7</td>
</tr>
<tr>
<td>EspD</td>
<td>EspD</td>
<td>+++</td>
<td>25.3 ± 1.8</td>
<td>36.9</td>
</tr>
<tr>
<td>Structural components</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EscR</td>
<td>EscR</td>
<td>+++</td>
<td>21.4 ± 2.6</td>
<td>43.1</td>
</tr>
<tr>
<td>EscS</td>
<td>EscR</td>
<td>+</td>
<td>3.3 ± 0.7</td>
<td>6.7</td>
</tr>
<tr>
<td>EscU</td>
<td>EscR</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>SepZ</td>
<td>EscR</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EscU</td>
<td>rOrf8</td>
<td>+</td>
<td>2.7 ± 0.1</td>
<td>6.3</td>
</tr>
<tr>
<td>EscC(mat†)</td>
<td>EscD</td>
<td>+++</td>
<td>7.8 ± 1.6</td>
<td>12.7</td>
</tr>
<tr>
<td>The EspD translocator and inner-membrane components</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rOrf1</td>
<td>EspD</td>
<td>+++</td>
<td>2.5 ± 0.2</td>
<td>3.9</td>
</tr>
<tr>
<td>EscR</td>
<td>EspD</td>
<td>+++</td>
<td>2.9 ± 0.1</td>
<td>4.9</td>
</tr>
<tr>
<td>EscS</td>
<td>EspD</td>
<td>+++</td>
<td>3.0 ± 0.5</td>
<td>5.1</td>
</tr>
<tr>
<td>EscU</td>
<td>EspD</td>
<td>+++</td>
<td>2.8 ± 0.6</td>
<td>5.7</td>
</tr>
<tr>
<td>SepZ</td>
<td>EspD</td>
<td>+++</td>
<td>3.9 ± 0.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Truncations of EspD‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EspD</td>
<td>EspD(N)</td>
<td>+++</td>
<td>12.0 ± 0.3</td>
<td>17.5</td>
</tr>
<tr>
<td>EspD</td>
<td>EspD(C)</td>
<td>+++</td>
<td>30.2 ± 0.1</td>
<td>44.1</td>
</tr>
<tr>
<td>CesD</td>
<td>EspD(C)</td>
<td>+++</td>
<td>22.6 ± 3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>rOrf1</td>
<td>EspD(C)</td>
<td>+</td>
<td>2.3 ± 0.7</td>
<td>3.6</td>
</tr>
<tr>
<td>EscR</td>
<td>EspD(C)</td>
<td>+</td>
<td>2.0 ± 0.3</td>
<td>3.3</td>
</tr>
<tr>
<td>EscS</td>
<td>EspD(C)</td>
<td>+</td>
<td>2.8 ± 0.2</td>
<td>4.8</td>
</tr>
<tr>
<td>EscU</td>
<td>EspD(C)</td>
<td>+</td>
<td>3.4 ± 0.6</td>
<td>6.8</td>
</tr>
<tr>
<td>SepZ</td>
<td>EspD(C)</td>
<td>+</td>
<td>2.1 ± 0.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Chaperones and secreted proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CesD</td>
<td>EspD</td>
<td>+++</td>
<td>15.9 ± 0.2</td>
<td>2.3</td>
</tr>
<tr>
<td>CesT</td>
<td>Map</td>
<td>+++</td>
<td>8.3 ± 0.4</td>
<td>19.4</td>
</tr>
<tr>
<td>Map</td>
<td>CesT</td>
<td>+++</td>
<td>51.2 ± 2.3</td>
<td>51.9</td>
</tr>
<tr>
<td>CesT</td>
<td>EspF</td>
<td>+</td>
<td>5.7 ± 0.1</td>
<td>10.3</td>
</tr>
<tr>
<td>Orf3</td>
<td>EspA</td>
<td>+++</td>
<td>11.9 ± 0.3</td>
<td>11.5</td>
</tr>
<tr>
<td>EspA</td>
<td>Orf3</td>
<td>+++</td>
<td>6.3 ± 0.3</td>
<td>10.3</td>
</tr>
<tr>
<td>Orf3</td>
<td>EspB</td>
<td>+</td>
<td>5.2 ± 0.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Other interactions</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SepL</td>
<td>rOrf6</td>
<td>+++</td>
<td>15.8 ± 0.3</td>
<td>8.2</td>
</tr>
<tr>
<td>rOrf6</td>
<td>SepL</td>
<td>+++</td>
<td>12.5 ± 0.6</td>
<td>10.1</td>
</tr>
<tr>
<td>Orf2</td>
<td>Orf29</td>
<td>+++</td>
<td>22.6 ± 1.9</td>
<td>29.6</td>
</tr>
<tr>
<td>Orf29</td>
<td>Orf2</td>
<td>+++</td>
<td>11.5 ± 0.4</td>
<td>14.2</td>
</tr>
<tr>
<td>Orf10</td>
<td>Orf10</td>
<td>+</td>
<td>3.4 ± 0.5</td>
<td>2.8</td>
</tr>
<tr>
<td>Orf11</td>
<td>Orf10</td>
<td>+</td>
<td>13.4 ± 0.8</td>
<td>17.0</td>
</tr>
<tr>
<td>Tir</td>
<td>Tir</td>
<td>+</td>
<td>3.2 ± 0.7</td>
<td>1.8</td>
</tr>
<tr>
<td>rOrf3</td>
<td>rOrf8</td>
<td>+</td>
<td>3.5 ± 0.1</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Fold increase over the β-galactosidase units of the strain expressing the bait alone added to those of the strain expressing the prey alone.
†The mature form of the protein, i.e. the protein without its N-terminal signal sequence.
‡EspD lacking the N-terminal (N) or C-terminal (C) hydrophobic domain.

et al., 1998; Kresse et al., 1999) and formation of the translocation pore (Ide et al., 2001) but has not previously been described to be associated with the TTSS apparatus. All the proteins that interact with EspD have predicted transmembrane domains and EscR, S and U are predicted to be located in the inner membrane based on homology.
with other systems (Hueck, 1998). No other secreted proteins were found to interact with the secretion apparatus, suggesting that binding to the inner-membrane components of the apparatus is a new function specific to EspD.

In order to investigate further binding of EspD to the structural inner-membrane proteins we used mutated forms of EspD that lack one of the two hydrophobic domains found within EspD (Fig. 2). These binding assays revealed that C-terminal hydrophobic domain of EspD is not required for interaction with any of the inner-membrane components, for EspD : EspD homo-protein interaction or for binding of EspD to its chaperone CesD (Table 4). In contrast, the N-terminal hydrophobic domain was found to be essential for the interactions with the inner-membrane proteins and with CesD (data not shown), but is dispensable for interaction with wild-type EspD (Table 4). We conclude that these interactions are specific because although the N-terminal hydrophobic domain of EspD is required for binding to the inner-membrane proteins, this phenomenon cannot be obtained by any hydrophobic domain, i.e. the C-terminal domain. In addition, the N-terminal domain is also required for the bona fide interaction with CesD.

(ii) Chaperones and secretion substrates. A third set of interactions involved proteins known to be secreted by the TTSS (Table 4). Some of these were interactions with their established chaperones (CesT : Tir, CesD : EspD) but others suggest new binding partners for established and putative chaperones (CesT : Map, CesT : EspF, Orf3 : EspA, Orf3 : EspB).

CesT is the chaperone for Tir (Abe et al., 1999; Elliott et al., 1999) and is known to form homodimers (Delahay et al., 2002); consequently CesT : CesT and CesT : Tir interactions were not unexpected. However, CesT was also found to interact strongly with another secreted protein, Map (Kenny & Jepson, 2000), which is encoded immediately upstream of Tir, and also weakly with EspF. The CesT : Map interaction has been further characterized and shown to be relevant and physiological, as EPEC exhibits CesT-dependent secretion and translocation of Map. This finding has been published elsewhere (Creasey et al., 2003).

Another particularly interesting interaction was that of Orf3 with both EspA and EspB. The interaction between Orf3 and EspA was observed with either protein as the bait but the interaction between Orf3 and EspB was only observed with Orf3 as the bait. These interactions were unexpected because previous studies have indicated that EspA in particular is unsuitable for study by the Y2H due to its propensity to form large aggregates (B. C. Neves and others, unpublished observations), thereby precluding interaction with any other protein. We hypothesized that Orf3 might perform a chaperone function for EspA, preventing premature aggregation within the bacterial cell and, by extrapolation, also within the yeast cell. This hypothesis has subsequently been confirmed using complementary biochemical approaches (E. A. Creasey and others, unpublished observations).

### Other interactions

The remaining novel interactions are difficult to characterize as they predominantly involve two uncharacterized proteins, which have no homologues in other TTSSs (Table 4); however, they are conserved among A/E pathogens, suggesting that they are important elements of LEE-encoded TTSSs. Among these interactions Orf10 was found to interact with itself and with Orf11; Orf10 and Orf11 have no homology with any other type III secretion proteins and similarly have no conserved domains that might allude to their function. It is worth noting that orf10 and orf11 do not belong to any of the five polycistronic operons and might form an independent transcriptional unit between LEE1 and LEE2 operons.

Although the SepL bait is a weak self-activator, when it is co-expressed with the rOrf6 prey the activation of the reporter genes is significantly enhanced (growth on selective media occurs 2–3 days earlier and β-galactosidase units are increased; see Table 4). Importantly there is also an interaction between the rOrf6 bait and the SepL prey, validating this result. Neither of these proteins has been well characterized and they are not conserved between TTSSs of different bacterial species. However, this protein interaction has been confirmed and characterized in detail; this study will be reported elsewhere (C. B. O’Connell and others, personal communication).

Other interactions involving proteins that remain uncharacterized include Orf2 : Orf29 and rOrf3 : rOrf8. rOrf3 has a putative signal peptide and transglycosylation domain, suggesting that it is secreted by the general secretion pathway and potentially functions in the periplasm to disrupt the peptidoglycan layer.

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**Table 5. Self-activating bait plasmids**

<table>
<thead>
<tr>
<th>Bait</th>
<th>Growth on selective plates</th>
<th>β-Galactosidase units (mean ± SD, n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CesD</td>
<td>+ +</td>
<td>6.7 ± 1.1</td>
</tr>
<tr>
<td>Orf12</td>
<td>+</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>SepQ</td>
<td>+ +</td>
<td>21.8 ± 3.3</td>
</tr>
<tr>
<td>Tir</td>
<td>+</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>SepL</td>
<td>+</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>EscF</td>
<td>+</td>
<td>1.2 ± 0.2</td>
</tr>
</tbody>
</table>

**Fig. 2.** Organization of the hydrophobic transmembrane domains (TM) in EspD.
Tir was found to interact with itself, although weakly. This was not unexpected as Tir was found as a dimer in the crystal structure of intimin–Tir complexes (Luo et al., 2000).

**DISCUSSION**

Many Gram-negative bacteria employ a specialized TTSS to deliver virulence effector proteins directly into the cellular environment of the eukaryotic host. While the effector proteins, as well as the host responses elicited, differ among TTSSs, they all utilize a conserved set of proteins that together form a bacterial-envelope-associated secretory organelle or needle complex (NC). These assemblies, which are elaborated on the bacterial cell surface, are large and complex, consisting of many different proteins and subunits. Assembly of the TTSS occurs through ordered secretion and polymerization events and requires the coordinated expression and association of over 20 different proteins.

The aim of this project was to identify novel interactions between proteins encoded by the LEE PAI, which encodes a TTSS. The LEE encodes a mixture of cytoplasmic proteins, secreted proteins and transmembrane proteins. Hydrophobic domains such as signal peptides and transmembrane domains can limit the usefulness of the standard Y2HS by inhibiting nuclear localization and possibly resulting in non-specific interactions between hydrophobic domains. In this study, we removed signal peptides to prevent their influence; however we took no specific action to compensate for the presence of transmembrane domains. All the proteins encoded by the LEE pathogenicity island were screened with the exception of Ler and intimin; in addition, EscV and EscN could not be cloned into the yeast two-hybrid vectors. All possible combinations of the remaining 37 proteins were screened, enabling us to identify novel interactions between TTSS components. Twenty-five interacting protein pairs were identified, of which 22 represented novel interactions (diagrammatically represented in Fig. 3).

**Structural proteins**

The NC consists of proteins that span the bacterial membrane, factors associated with the inner membrane and an external needle. In addition EPEC also has a long filament attached to the distal end of the needle. Many of these proteins are highly conserved between virulence TTSSs and the flagellar export system, suggesting a common structure. Therefore interactions identified in this screen which involve conserved proteins may be relevant for other type III systems.

![Diagram of TTSS components](image-url)
Although most of the inner-membrane-associated proteins have been identified for all systems little is known about how they assemble and function within the bacterial cell. Some of the interactions identified in this project involve combinations of inner-membrane proteins and may provide an initial idea about the structure of this part of the secretion apparatus. Based on homology and structural predictions, eight LEE-encoded proteins are thought to span the inner membrane: EscR, S, T, U, V, D, J and SepZ. With the exception of SepZ, these proteins are highly conserved among TTSSs. EscR is predicted to have four transmembrane domains as does its homologue in Yersinia, YscR (Hueck, 1998), and it interacts strongly with itself, suggesting that EscR is present in the membrane as a multimer. We also found that EscR interacts with three other membrane-spanning proteins: EscS, EscU and SepZ. EscS is predicted to have two transmembrane domains as do its homologues (Hueck, 1998). SepZ is also predicted to have two transmembrane domains but does not have homologues in other TTSSs. EscU and its homologues are predicted to have four transmembrane domains in their amino terminus (Allaoui et al., 1994) and a carboxy-terminal cytoplasmic domain implicated in substrate specificity switching (Williams et al., 1996; Minamino & Macnab, 2000b). These interactions suggest that EscR, S, U and SepZ form a complex within the bacterial inner membrane. We did not detect any interactions involving EscT, EscJ or EscD and other inner-membrane proteins, possibly as a result of incorrect folding in the yeast nucleus or because stable interactions are only formed in the presence of more than one other protein or in the context of the specific local environment. In addition we have so far been unable to clone EscV into the yeast two-hybrid vectors; therefore no interactions involving EscV were identified. However, these proteins are predicted to span the inner membrane and are therefore likely to be involved in the inner-membrane protein complex. Indeed, the Salmonella homologues of all of these proteins were shown to be required for NC formation (Sukhan et al., 2001).

In EPEC, only one protein, EscC, is known to be associated with the outer membrane. EscC and its homologues are members of the secretin family of proteins, which are involved in the transport of various macromolecules and filamentous phages across the outer membrane (Genin & Boucher, 1994). These proteins form a multimeric ring in the outer membrane and are likely to form a channel through which secreted proteins pass. However, in this study we did not detect an EscC:EscC homo-protein interaction, and it is possible that the formation of the outer-membrane ring requires the cooperation of other LEE-encoded proteins; indeed, the EscC family member, PulD, and some of the TTSS homologues of EscC require ‘pilot’ proteins for their stable integration into the outer membrane (PulD: PulS, Nouwen et al., 1999; MxiD: MxiM, Schuch & Maurelli, 2001). As yet no pilot protein has been identified for the EPEC system. Electron micrographs of NCs show that the outer-membrane rings are connected to the inner-membrane rings by a cylindrical structure. A study of the Shigella NC using the Y2HS showed that the periplasmic domains of MxiD (EscC) and MxiJ (EscJ) interact (Schuch & Maurelli, 2001). In this study, no interaction was detected between EscJ and EscC; however we did detect an interaction between EscC and the inner-membrane protein EscD. This interaction may suggest a structural difference between the EPEC and Shigella NCs. Indeed Shigella lacks an EscD homologue.

Cytoplasmic proteins

As well as structural components, TTSSs also have soluble factors including chaperones, secreted proteins and regulators. The LEE-encoded regulator, Ler, was not included in this study because of its ability to activate transcription; however all other LEE-encoded proteins predicted to be soluble were included.

The Y2HS has been used widely to study interactions between secreted proteins and their cognate chaperones (Elliott et al., 1999; Francis et al., 2000, 2001; Delahay et al., 2002; Page et al., 2002). To date four LEE-encoded chaperones have been identified: CesT (Abe et al., 1999; Elliott et al., 1999), CesD (Wainwright & Kaper, 1998), CesD2 (Neves et al., 2003) and CesF (Elliott et al., 2002), which perform chaperone functions for Tir, EspD/B and EspF, respectively. Interactions between CesT and Tir and between CesD and EspD were detected in this study as expected. However, we were unable to detect an interaction between CesD2 and EspD or between CesF and EspF using the Y2HS approach.

In addition to the expected chaperone interactions we also detected two further binding partners of CesT, Map and EspF. In a separate paper we described in detail the characteristics and physiological consequences and significances of the CesT:Map interaction (Creasey et al., 2003). The weak interaction between CesT and EspF identified in this screen has not yet been followed.

Two further interactions were detected involving secreted proteins. Orf3 was found to interact with both EspA and EspB. The interaction between EspA and EspB is not detectable using the Y2HS (Hartland et al., 2000), possibly due to the propensity of EspA to form large aggregates. We hypothesize that Orf3 is a chaperone preventing premature aggregation of EspA and/or interaction with EspB, roles that have been described for other chaperones (Neyt & Cornelis, 1999; Tucker & Galan, 2000; Auveray et al., 2001). Orf3 has no homologues in other TTSSs, although in common with other TTSS chaperones, it is predicted to be a small (12.3 kDa) mainly α-helical protein. However, unlike the majority of TTSS chaperones, Orf3 has an alkaline, rather than acidic pI (9.6), and was found not to interact with itself in the yeast screen, whereas many type III chaperones homodimerize. In addition, orf3 is the only gene missing from the RDEC-1 LEE (Zhu et al., 2001), suggesting that if it is important for the EPEC system, it is not necessary for all
LEE-encoded TTSSs. We have investigated the physiological significance of the Orf3 : EspA and Orf3 : EspB interactions in greater depth and will report our findings in a separate paper (E. A. Creasey and others, unpublished).

Many of the uncharacterized LEE-encoded proteins are predicted to be soluble proteins, possibly located in the bacterial cytoplasm, and several interactions were identified involving these proteins: Orf2 : Orf29, SepL : rOrf6, Orf10 : Orf11, Orf10 : Orf15 and rOrf3 : rOrf8. None of these proteins have homologues in other TTSSs although they are conserved among A/E pathogens, suggesting that they play an important role in pathogenesis. The interaction between SepL and rOrf6 (now called SepD) has been characterized further and will be described elsewhere (C. B. O’Connell and others, personal communication).

**Structural proteins and the EspD translocator protein**

EspD is an EPEC translocator protein that forms a translocation pore in the host membrane (Ide et al., 2001) in addition to its role in EspA filament biogenesis (Kresse et al., 1999). The identification of EspD : EspD and EspD : CesD protein interactions confirmed previous reports (Wainwright & Kaper, 1998; Daniell et al., 2001); however, EspD was unexpectedly found to interact with a complement of membrane-spanning proteins, including EscR, EscS, EscU, SepZ and Rorf1. The membrane localization of EspD has been previously reported, and the EspD chaperones, CesD and CesD2, were similarly shown to be mainly membrane-associated (Wainwright & Kaper, 1998; Neves et al., 2003), indicating the precedent for interaction of EspD with membrane components of the TTS apparatus.

It is clear that there is a hierarchy involved in the passage of proteins through the TTSS. Structural subunits are initially exported to complete assembly of the TTSS on the surface of the bacterium, which allows subsequent passage of the translocators to the host membrane, and the effectors to the host cytosol. A similar hierarchical structure is seen in the flagellar system, where secretion is switched from rod/ hook-type proteins to filament-type proteins (Minamino & Macnab, 1999). In the light of these interactions it is tempting to speculate that EspD ‘docks’ at the inner membrane of the secretion apparatus before secretion is initiated and that EspD is the first secreted protein to leave the bacterial cell. Indeed in the absence of EspD, the secretion of the other translocators is reduced and EspA filaments are not formed (Kresse et al., 1999; Daniell et al., 2001).

Although a powerful tool, the Y2HS is subject to inherent limitations, in the form of false-positive and false-negative results. In order to reduce the frequency of false-positive returns, we used the yeast host strain PJ69-4A, specifically designed for that purpose, comprising three unlinked reporter genes, each under the control of an independent promoter (James et al., 1996). Additionally, scoring the results of two separate phenotypes indicative of a positive interaction (growth on minimal medium and β-galactosidase activity) was deemed to increase the overall confidence of a positive interaction. However, despite these advances, we were vigilant throughout this study to identify auto-activating constructs (Table 5), and hence interactions involving self-activating AD or BD fusions were interpreted with caution.

We were also concerned that some of the interactions involving proteins with hydrophobic transmembrane domains may have resulted from non-specific aggregation in the hydrophilic yeast nucleus. This could potentially be a factor when accounting for the larger than expected subset of EspD interactions, although our preliminary deletion analysis suggests otherwise.

In general, it is necessary to validate Y2HS interactions using complementary biochemical approaches to rule out the possibility of false-positive interactions. Consequently a wider programme of research has been initiated to confirm and further explore some of the interactions identified by this screen (data not shown); however, in the absence of supporting data the novel interactions reported here should be viewed with appropriate caution.

In addition to potential false-positive returns, false-negative results were also anticipated. Indeed the propensity of many LEE-encoded proteins such as EscC, EscF, and most notably EspA, to homo-oligomerize would effectively preclude detection of their interactions in this system, as a consequence of either inefficient targeting to the nucleus or the blocking of productive interactions. This has previously been noted for the expected EspA : EspB (Hartland et al., 2000), and EspA : EspA (B. C. Neves and others, unpublished observations), interactions.

False negatives may also occur for many other reasons, for example, interactions with equilibrium dissociation constants lower than the cut-off of 10–50 μM cannot be detected and interactions that involve immediate aminoterminal sequences of proteins may be inhibited because of steric constraints arising from the adjacent Gal4 domain in the hybrid proteins. Similarly, low expression, the absence of post-translational modification events which predispose protein interactions, or aberrant protein folding in the yeast background all contribute to false-negative returns for pair-wise interactions.

Incorrect protein folding or inefficient nuclear targeting in the yeast cell can also arise due to the presence of hydrophobic regions such as transmembrane domains and sec-dependent signal peptides within the target proteins. We have limited the affect of sec-dependent signal peptides by cloning the mature forms of LEE proteins; however, many of the proteins included in this study have one or more transmembrane domains and no steps have been taken to limit the effect of these. Although Y2HSs have been...
developed to study membrane-bound proteins (Drees, 1999) we chose to use a standard system because of the homogeneity of protein type encoded by the LEE. For a more valid study of membrane-bound TTSS proteins alternative Y2HS approaches would be more suitable. Alternatively, screening a random hybrid library would allow detection of interactions mediated by less hydrophobic domains of transmembrane proteins, and similarly, loss of cryptic activation/binding domains or more specific DNA binding segments of auto-activators would facilitate detection of interactions mediated by these proteins.

Despite these limitations the Y2HS does provide an effective discovery method and has been used widely in the study of TTSSs. Although the specificity of many interactions reported here requires further confirmation, and several LEE proteins were necessarily omitted from the study, this investigation remains the most comprehensive Y2HS screen to date, applied to investigate pair-wise protein interaction between LEE-encoded proteins. Hence, the putative interactions identified in this study provide further insights into the structural assembly of the EPEC TTSS and the events that occur to facilitate secretion of substrates. These observations may also reflect on other virulence TTSSs, given the conservation of many of the components involved. Additional potential interactions involving proteins unique to the LEE pathogens also highlight differences that may have evolved to accommodate individual pathogenic strategies. Accordingly, this study provides a platform on which to generate hypotheses for further investigation of the TTSS assemblies.

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REFERENCES


