Dissection of homologous translocon operons reveals a distinct role for YopD in type III secretion by Yersinia pseudotuberculosis

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The homologous pcrGVHpopBD and lcrGVHyopBD translocon operons of Pseudomonas aeruginosa and pathogenic Yersinia spp., respectively, are responsible for the translocation of anti-host effectors into the cytosol of infected eukaryotic cells. In Yersinia, this operon is also required for yop-regulatory control. To probe for key molecular interactions during the infection process, the functional interchangeability of popBlipopB and popDyopD was investigated. Secretion of PopB produced in trans in a ΔyopB null mutant of Yersinia was only observed when co-produced with its native chaperone PcrH, but this was sufficient to complement the yop translocation defect. The Yersinia ΔyopD null mutant synthesized and secreted PopD even in the absence of native PcrH, yet this did not restore YopD-dependent yop-regulatory control or effector translocation. Thus, this suggests that key residues in YopD, which are not conserved in PopD, are essential for functional Yersinia type III secretion.

INTRODUCTION

All pathogenic Yersinia spp. encode a wide assortment of virulence determinants to establish a host infection (Revell & Miller, 2001). Significantly, a common plasmid-borne type III secretion system (TTSS) is essential for the fitness of Yersinia inside the host (Cornelis et al., 1998). This specialized virulence strategy links protein secretion across the bacterial envelope with translocation of anti-host virulence effectors through the plasma membrane of target eukaryotic cells. Effector proteins localized inside the target cell disable crucial signal transduction pathways, rendering the cell incapable of mounting an effective immune defence (Bliska, 2000; Fällman et al., 2002).

The bacterial envelope component of the TTSSs of many plant- and animal-interacting bacteria resembles the flagella basal body, while a hollow needle-like protrusion extends from the bacterial surface through which effectors are believed to traverse (Blocker et al., 1999; Hoiczyk & Blobel, 2001; Jin & He, 2001; Kubori et al., 1998; Sekiya et al., 2001; Tamano et al., 2000). Furthermore, translocation into target cells by Yersinia requires the structural proteins LcrV, YopB and YopD. These proteins interact (Neyt & Cornelis, 1999b; Sarker et al., 1998) to presumably form a complex that facilitates pore formation in target cell membranes to allow entry of effector proteins into target cells (Bröms et al., 2003a; Holmström et al., 2001; Neyt & Cornelis, 1999a; Tardy et al., 1999). At odds with this dogma is a report suggesting that the needle component YscF is the sole requirement for effector translocation (Hoiczyk & Blobel, 2001). Clearly, the molecular mechanisms of this process are still unresolved. Interestingly, both LcrV and YopD also contribute to the control of type III substrate synthesis and secretion. Yop synthesis in LcrV-defective strains is significantly down-regulated (Bergman et al., 1991; Price et al., 1991; Skrzypek & Straley, 1995). On the other hand, Yersinia defective for YopD constitutively produce Yop proteins and LcrV (Francis & Wolf-Watz, 1998; Williams & Straley, 1998). However, this phenotype depends on a complex between YopD and LcrH (Anderson et al., 2002; Francis et al., 2001), a specialized type III secretion chaperone that ensures the pre-secretory stabilization and efficient secretion of both YopB and YopD (Francis et al., 2000; Neyt & Cornelis, 1999b; Wattiau et al., 1994). These regulatory and translocon components are encoded by the lcrGVHyopBD polycistronic translocase operon, reflecting their common functional themes (Bergman et al., 1991).

Pseudomonas aeruginosa also utilizes a TTSS to establish a variety of acute opportunistic infections (Lyczak et al., 2000). This system is genetically and structurally similar to the plasmid-encoded TTSS of Yersinia (Frank, 1997;
Hueck, 1998), which might reflect a common preference to remain extracellular during an infection. Central to this study is the fact that *P. aeruginosa* also possesses a homologous translocase operon (*pcrGVHpopBD*) (Fig. 1) (Yahr et al., 1997). Like the LcrH chaperone of *Yersinia*, PcrH is responsible for pre-secretory stabilization and efficient secretion of PopB and PopD by *P. aeruginosa* (Bröms et al., 2003b). Furthermore, *P. aeruginosa* PcrV, PopB and PopD exhibit analogous functions with respect to effector translocation into eukaryotic cells (Bröms et al., 2003a; Dacheux et al., 2001; Sawa et al., 1999; Sundin et al., 2002). It follows that expression of the full *pcrGVHpopBD* operon in a ΔlcrGVHyopBD null mutant of *Yersinia* can efficiently restore wild-type levels of effector translocation (Bröms et al., 2003a). However, while PcrV alone can functionally complement a *Yersinia* ΔlcrV null mutant for regulation (Bröms et al., 2003a; Pettersson et al., 1999), efficient effector translocation clearly requires co-production of additional *P. aeruginosa* translocon components (Bröms et al., 2003a). In light of this finding, the fact that PopB and PopD could complement reciprocal defects in YopB and YopD of *Yersinia* when the entire *P. aeruginosa* translocase operon was used in the complementation study (Frithz-Lindsten et al., 1998) could be misleading. Efforts to complement using individual genes were hitherto not performed. Moreover, whether PopD could complement the regulatory defects observed for a ΔyopD null mutant of *Yersinia* was not addressed. Therefore, to further probe for key molecular mechanisms within this common *P. aeruginosa* and *Yersinia* virulence strategy, we investigated whether PopB and PopD could functionally complement the corresponding ΔyopB and ΔyopD null mutants of *Yersinia*. Our data suggest that key motifs within YopD modulate regulatory and translocation processes specific to type III function in *Yersinia*, since heterologous protein interactions that normally govern the regulation and translocation process are either non-functional or non-existent.

**METHODS**

**Strains, plasmids, culture media and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were grown routinely in Luria–Bertani broth (LB) or on blood agar base plates (BAB; Merck) at 26 °C (*Yersinia pseudotuberculosis*) or 37 °C (*Escherichia coli*) with aerlation. Where appropriate, antibiotics were used at the following concentrations: ampicillin (100 μg ml⁻¹), kanamycin (50 μg ml⁻¹), gentamicin (20 μg ml⁻¹) and chloramphenicol (25 μg ml⁻¹).

**Recombinant DNA techniques.** Plasmid DNA was isolated from *E. coli* using the High Pure Plasmid Purification kit (Roche Applied Science). Standard DNA manipulation techniques including use of restriction enzymes, *in vitro* DNA cloning, DNA mobilization and agarose gel electrophoresis were performed essentially as described by Sambrook et al. (1989). Recovery of DNA fragments from agarose was achieved by using GeneClean II (Bio 101) or Genelute minus EtBr spin columns (Sigma) as detailed by the manufacturer. All oligonucleotides for DNA amplification were purchased from MWG-Biotech AG (Germany).

**Construction of yopB deletion mutant.** Generation of the mutagenesis vector pMF463 used to construct the in-frame ΔyopB mutant YPIII/pBl615 in *Y. pseudotuberculosis* has been described elsewhere (Bröms et al., 2003a). pMF463 was maintained in *E. coli* S17-1λpir for conjugal mating experiments with *Y. pseudotuberculosis*. Allelic exchange between the pMF463 suicide plasmid and the complementary *yopB* gene of *Y. pseudotuberculosis* was performed as described previously (Milton et al., 1996). The engineered mutant strain was verified by PCR, DNA sequencing and Western blot analysis.

**Construction of plasmids for complementation of Yersinia mutants.** The PCR conditions employed to amplify DNA fragments from *P. aeruginosa* PAK included the use of DyNAzyme II DNA polymerase (Finnzymes) and 1× to 2.5× PCR Enhancer Solution (Invitrogen life technologies). Primers were designed from regions of absolute identity between sequences obtained from strain 388 (GenBank accession no. AF010149) (Yahr et al., 1997) and the *Pseudomonas* Genome Project using strain PAO1 (Stover et al., 2000). Products were initially cloned into the pCR4-TOPO cloning vector (Invitrogen) and sequenced using the DYEnamic ET terminator cycle sequencing kit (Amersham Biosciences) and an ABI Prism 377 DNA sequencer (Applied Biosystems). Each confirmed allele was subsequently subcloned into pMMB66HE or pMMB66EH (Fürste et al., 1986). To clone popB, the amplified DNA fragment was generated with the primer pair PopB3-F (5′-GCA TGT AAG CTT GAA GCC GTA ACC CGG AGA AAG GAT-3′) (HindIII) and PopB2-R (5′-GCA TGT GAA TTC TCA GAT CGC TGC CGG TC-3′) (EcoRI) to generate a HindIII–EcoRI DNA fragment for cloning (resulting expression plasmid termed pJEB49). The popD allele (pJEB44) was generated on a SalI–EcoRI DNA fragment using primers PopD3-F (5′-GCA TGT GTC GAC ATC TCA GGA GAC GTC ACA TGA-3′) (SalI) and PopD2-R (5′-GCA TGT GAA TTC AGA CGG TCT AGA CCA CT-3′) (EcoRI). The *pcrGVHpopBD* alleles (pJEB51) were simultaneously cloned by HindIII/EcoRI digestion of

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**Fig. 1. Schematic representation of the translocase operons of pathogenic *Yersinia* (lcrGVHyopBD) and *P. aeruginosa* (*pcrGVHpopBD*). The amino acid identities shared by the corresponding products are indicated in the centre and were obtained by pairwise alignment with the EMBOSS alignment tool (http://www.ebi.ac.uk/emboss/align). The solid lines indicate the expression constructs derived from *Yersinia* (above) or *P. aeruginosa* (below) that were used for complementation experiments. The broken lines illustrate deletions of the null mutants of *Yersinia* that were used in this study.
the amplified DNA generated with the primer combination of PcrH-F (5'-GCA TGT AGC GTT ATC GGA TT-3') and PopB2-R (EcoRI). The construct for co-expression of pcrHpopD (pJEB53) was achieved by overlap PCR (Horton & Pease, 1991) using the primer pair combinations of PcrH-F (5'-CGG AGA GAT CTG AGG AGA-3') and PopB2-R (EcoRI). The yopD allele was isolated from YPIII/pIB621 as template and primers lcrH1-F (5'-GCA TGT AGC GTT ATC GGA TT-3') and c (5'-GAT AAC GTA ATT ATG CAA-3') with PopD2 (EcoRI). The yopD allele was isolated from BamHI/PstI-digested pMF367 (Francis et al., 2000) and subcloned into pMM666EH, resulting in pJEB54. Generating the construct for co-expression of lcrHpopD (pJEB81) occurred by first amplifying lcrH by PCR using Y. pseudotuberculosis YPIII/pIB102 as template and primers LcrH1-F (5'-ACT ACT GAA TTC TGA CAC GAG GTA ATT ATG CAA C-3') (EcoRI) and LcrH1-R (5'-GCA GGA GAA GCC CAC GTC ACC AAC CAC GCA ATC GGA TT-3') (BamHI). This amplified lcrH allele was cloned into EcoRI/BamHI-digested pMM666EH, generating pJEB75 (J. E. Bröms, unpublished data). Subsequently, the yopD allele was cloned from BamHI/PstI-digested pMF367 and cloned downstream of lcrH in pJEB75, generating pJEB81. All alleles have been cloned with their native ribosome-binding site. Chemical transformation into E. coli was performed as described previously (Hanahan, 1983), as was the method of electroporation into Y. pseudotuberculosis (Dower et al., 1988).

**Growth phenotypes.** To determine the plating frequencies of the various *Yersinia* strains under high- and low-Ca$^{2+}$ conditions at 37°C, a MOX (magnesium oxide) analysis was performed twice, essentially as described by Bergman et al. (1991). For the analysis of *trans*-complemented strains in which the alleles are under an IPTG-responsive promoter, IPTG was added to a final concentration of 0.4 mM. Essentially, the plating frequency reflects the ability to form colonies at 37°C on agar plates with or without Ca$^{2+}$ compared to growth at 26°C. The frequency is given as a ratio of the number of colonies growing at 37°C compared to the number growing at 26°C. Normal regulatory control correlates to Ca$^{2+}$-dependent growth, while a loss of negative regulatory control correlates with the inability to grow at 37°C regardless of Ca$^{2+}$ levels.

**Analysis of synthesis of type III secreted substrates.** Induction of type III substrate synthesis and secretion from *Y. pseudotuberculosis* was performed using standard methods (Bergman et al., 1991), except that to induce expression from constructs under the control of the *pas* promoter, IPTG was added to a final concentration of 0.4 mM at the time of temperature upshift during cultivation. Western blot analysis of total protein levels sampled directly from the bacterial culture suspension (containing a mix of proteins at a concentration of 0.4 mM) was performed as described previously (Francis & Wolf-Watz, 1998). TCA-precipitated cleared supernatant samples containing only proteins secreted into the culture medium were fractionated by SDS-PAGE.
and detected by Coomassie brilliant blue staining. Bio-Rad pre-stained low range protein molecular mass standards were used in this analysis. Each experiment was repeated at least twice.

Cultivation and infection of HeLa cells. The human epithelial cell line HeLa was used in all in vitro infection experiments. Culture maintenance and infections with Yersinia followed established protocols (Sundin et al., 2001), except that IPTG (0–4 mM) was added to bacteria and cell monolayers prior to infection. The cytotoxicity of infected HeLa cells was monitored by light microscopy and images were collected at successive time points. Reproducibility of results was confirmed by performing at least three independent experiments.

RESULTS

PopB of P. aeruginosa and YopB of Y. pseudotuberculosis are functionally equivalent

It is noteworthy that only in the presence of other native P. aeruginosa translocon components can PcrV efficiently complement translocation defects attributed to a ΔlcrV null mutant of Yersinia (Bröms et al., 2003a). With this in mind, we wondered whether an earlier study showing efficient complementation of ΔyopB and ΔyopD mutants of Yersinia by the translocon operon of P. aeruginosa could be misleading (Frithz-Lindsten et al., 1998). Therefore, to directly test the functional conservation of PopB and YopB, we generated a near full-length ΔyopB null mutant allele in which only the first 12 and last two codons of YopB remained (pMF463) (Bröms et al., 2003a) in wild-type Yersinia creating YPIII/pIB615 (see Fig. 1). As anticipated, this mutant displays a wild-type pattern of Yop synthesis except for the notable absence of a band representing YopB (Fig. 2, compare lanes a and b with c and d). This protein profile is consistent with YopB not being involved in maintaining yop-regulatory control (Table 2) (Hartland et al., 1996; Håkansson et al., 1996).

To assess whether PopB could function in the Yersinia translocation machinery without its native translocator partners, we cloned popB (pJEB49) or pcrH and popB (pJEB51) under the control of an IPTG-inducible promoter and transformed them individually into the ΔyopB null mutant (YPIII/pIB615). Interestingly, only when co-produced with its native chaperone PcrH was PopB stabilized and secreted by the ΔyopB null mutant of Yersinia, albeit at low levels (Fig. 2, compare lanes h and l). Nonetheless, we wondered whether secreted PopB could function in concert with endogenous Yersinia proteins to establish Yop-effector translocation. This can be easily visualized by performing a tissue culture cytotoxicity assay. When translocated, the YopE GTPase-activating protein of the Rho-family of small G-proteins will depolymerize host cell actin, causing a general rounding up of infected cells (Rosqvist et al., 1991). Not surprisingly, YPIII/pIB615 was

Fig. 2. Analysis of Yop/Pop synthesis and secretion from Y. pseudotuberculosis strains grown either with (+) or without (−) Ca²⁺. Yop/Pop proteins were separated by SDS-PAGE and identified either by immunoblot analysis (upper panel) using polyclonal rabbit anti-YopH, anti-YopB (cross-reacts with PopB), anti-LcrV and anti-YopD antiserum (total protein fraction – a mix of Yop/Pop proteins secreted to the culture medium and contained within intact bacteria) or by Coomassie brilliant blue staining (lower panel) of TCA-precipitated secreted proteins (derived from cleared culture supernatants). Where indicated, IPTG was added at a final concentration of 0.4 mM upon temperature shift. Lanes: a and b, wild-type YPIII/pIB102; c and d, ΔyopB null mutant YPIII/pIB615; e–h, complemented YPIII/pIB615(pJEB49); i–l, complemented YPIII/pIB615(pJEB51). Molecular masses, shown in parentheses, were deduced from primary sequence. The single asterisk highlights low-level secretion of PopB only when co-produced with its native chaperone PcrH. The double asterisk indicates a protein band that cross-reacts with the anti-LcrV antiserum.
Table 2. Growth phenotypes and plating frequencies of Yersinia strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype/phenotype</th>
<th>Plating frequency*</th>
<th>Growth phenotype†</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>With Ca²⁺</td>
<td>Without Ca²⁺</td>
</tr>
<tr>
<td>YPIII/pIB102</td>
<td>Wild-type</td>
<td>1</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB615</td>
<td>ΔyopB</td>
<td>1</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB621</td>
<td>ΔyopD</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB621(pJEB44)</td>
<td>ΔyopD pPopD⁺</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB621(pJEB54)</td>
<td>ΔyopD pYopD⁺</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB621H</td>
<td>ΔlcrH yopD⁺</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
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<tr>
<td>YPIII/pIB621H(pJEB44)</td>
<td>ΔlcrH yopD pPopD⁺</td>
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<tr>
<td>YPIII/pIB621H(pJEB54)</td>
<td>ΔlcrH yopD pYopD⁺</td>
<td>10⁻⁴</td>
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<tr>
<td>YPIII/pIB621H(pJEB53)</td>
<td>ΔlcrH yopD pPcrH PopD⁺</td>
<td>10⁻⁴</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>YPIII/pIB621H(pJEB91)</td>
<td>ΔlcrH yopD pLcrH YopD⁺</td>
<td>1</td>
<td>10⁻²</td>
</tr>
</tbody>
</table>

*Plating frequency readout derived from MOX analysis of Yersinia mutants trans-complemented with popD, yopD, pcrH and lcrH alleles under the control of an IPTG-inducible promoter. Determinations were made following at least two independent experiments according to the protocol of Bergman et al. (1991).

†CD, Calcium dependent – bacteria grow only in the presence of Ca²⁺ at 37 °C, reflecting wild-type regulatory control of Yop synthesis; TS, temperature sensitive – bacteria are growth-restricted at 37 °C, reflecting defective regulatory control whereby Yop synthesis is constitutive.

unlable to induce a YopE-dependent cytotoxic response when used to infect HeLa cell monolayers (Fig. 3B), consistent with the requirement for YopB in effector translocation (Hartland et al., 1996; Häkansson et al., 1996; Nordfelth & Wolf-Watz, 2001). In contrast, however, only when this strain secreted PopB (YPIII/pIB615, pJEB51) did a rapid cytotoxic response ensue that mirrored infection only when this strain secreted PopB (YPIII/pIB621H(pJEB44)) (compare D with A). This occurred even though marginal PopB secretion was actually observed in vitro (see Fig. 2, lower panel, lane 1).

According to computer predictions, YopB and PopB both exhibit two potential hydrophobic transmembrane domains in the central regions of each protein (Fig. 4) (Häkansson et al., 1993) and coiled-coil protein–protein interaction domains near the N terminus and the C terminus (Fig. 4) (Delahay & Frankel, 2002; Pallen et al., 1997). In addition, IpaB from Shigella sp., which overall displays a modest 15% identity to YopB, has also been ascribed this same structural architecture that befits a member of this translocator subfamily (Fig. 4) (Delahay & Frankel, 2002; Häkansson et al., 1993; Pallen et al., 1997; Wachtet al., 1999). Not surprisingly, the amino acid relatedness of these three proteins was greater within the putative transmembrane and coiled-coil domains (Fig. 4). Thus, to determine if these conserved motifs per se are sufficient to promote translocation in Yersinia, we introduced IpaB co-produced with its cognate chaperone IpgC (Menard et al., 1994) under the control of an inducible promoter (pSEB2) (Rosqvist et al., 1995) into the ΔyopB null mutant of Yersinia. Even though it was secreted (Fig. 5) (Rosqvist et al., 1995), IpaB did not restore the translocation defect associated with the loss of YopB (Fig. 3E). This indicates that the conserved structural motifs, which define this protein subfamily (Fig. 4), are not the sole requirement for efficient PopB complementation of a ΔyopB null mutant. Rather, YopB and PopB, but not IpaB, must contain other regions of sufficient identity, which are located outside of the putative transmembrane and coiled-coil domains that permit functional conservation. This is reflected by the more extensive relatedness of YopB and PopB, compared to IpaB (Fig. 4). This clarifies an earlier report (Fritzh-Lindsten et al., 1998) that PopB from P. aeruginosa, when secreted by Y. pseudotuberculosis, is truly functionally analogous to YopB, which reinforces the close relatedness between the Yersinia and P. aeruginosa TTSSs.

**P. aeruginosa** _PopD_ fails to restore translocation by _Y. pseudotuberculosis_ defective for _YopD_

By analogy with the preceding experiments, we wanted to determine if PopD could complement defective YopD of _Yersinia_ when expressed in isolation from other native _P. aeruginosa_ translocon partners. To investigate this aspect, we took advantage of the previously characterized ΔyopD (YPIII/pIB621) (Francis & Wolf-Watz, 1998) or ΔlcrH yopD (YPIII/pIB621H) (Francis et al., 2000) null mutants of _Y. pseudotuberculosis_. These mutants exhibit a pleiotropic phenotype having lost yop- regulatory control (constitutive Yop production ensues) and the ability to translocate Yop-effectors into the cytosol of target cells. We introduced the _P. aeruginosa_ alleles popD (pJEB44) or popD and pcrH (pJEB53) under the control of an IPTG-inducible promoter into the ΔyopD or ΔlcrH yopD null mutants, respectively. From these strains, total protein and protein secreted into the culture media were fractionated by SDS-PAGE and visualized by immunoblot (Fig. 6, upper panel) and Coomassie brilliant blue staining (Fig. 6, lower panel), respectively. This revealed that PopD was readily stabilized...
and secreted by *Y. pseudotuberculosis* in the presence of heterologous LcrH (Fig. 6, lane h). However, PopD clearly requires a chaperone for stability (Fig. 6, upper panel, lane r). Notably, the level of PopD secretion was not significantly improved when co-produced with the *ΔyopD* deletion mutant or when complemented with either pJEB49 (PopB⁺) or pSEB2 (lpgC⁺ lpaB⁺) show normal cell morphology, as do infections with the *ΔyopD* deletion mutant or when complemented with pJEB44 (PopD⁺), pJEB53 (PcrH⁺ PopD⁺) or pJEB44, pTS103-Gm (PopD⁺ ExoS⁺) (compare L with B, C and E–I). Shown are phase-contrast images of cells infected with wild-type YPIII/pIB102 (A), *ΔyopB* null mutant YPIII/pIB615 (B), complemented YPIII/pIB615 (pJEB49) (C), complemented YPIII/pIB615 (pJEB51) (D), complemented YPIII/pIB615 (pSEB2) (E), *ΔyopD* null mutant YPIII/pIB621 (F), complemented YPIII/pIB621 (pJEB44) (G), complemented YPIII/pIB621 (pJEB53) (H), complemented YPIII/pIB621 (pJEB44, pTS103-Gm) (I), complemented YPIII/pIB621 (pJEB64) (J), complemented YPIII/pIB621 (pJEB91) (K) and uninfected (L).

**Fig. 3.** Infection of HeLa cells by *Y. pseudotuberculosis*. Strains were allowed to infect monolayers and, after 3 h post-infection, the effect of the bacteria on the HeLa cells was recorded by phase-contrast microscopy. Note the extensive rounding up of the YopE-dependent, cytotoxically affected HeLa cells (A, D, J and K). HeLa cells infected with the *ΔyopB* deletion mutant or when complemented with either pJEB49 (PopB⁺) or pSEB2 (lpgC⁺ lpaB⁺) show normal cell morphology, as do infections with the *ΔyopD* deletion mutant or when complemented with pJEB44 (PopD⁺), pJEB53 (PcrH⁺ PopD⁺) or pJEB44, pTS103-Gm (PopD⁺ ExoS⁺) (compare L with B, C and E–I). Shown are phase-contrast images of cells infected with wild-type YPIII/pIB102 (A), *ΔyopB* null mutant YPIII/pIB615 (B), complemented YPIII/pIB615 (pJEB49) (C), complemented YPIII/pIB615 (pJEB51) (D), complemented YPIII/pIB615 (pSEB2) (E), *ΔyopD* null mutant YPIII/pIB621 (F), complemented YPIII/pIB621 (pJEB44) (G), complemented YPIII/pIB621 (pJEB53) (H), complemented YPIII/pIB621 (pJEB44, pTS103-Gm) (I), complemented YPIII/pIB621 (pJEB64) (J), complemented YPIII/pIB621 (pJEB91) (K) and uninfected (L).

Since PopD was readily secreted by *Y. pseudotuberculosis*, we investigated if this protein could complement the translocation defect normally exhibited by a *ΔyopD* null mutant (Francis & Wolf-Watz, 1998; Rosqvist *et al.*, 1994). PopD
Analysis of IpaB synthesis and secretion from Fig. 5.

Grey) were identified using the COILS web server (http://www.ch.embnet.org/software/COILS_form.html) and membrane-spanning regions (TM, light grey) were predicted using the TMPRED web server (http://www.ch.embnet.org/software/TMPRED_form.html). A pairwise alignment of YopB with PopB or IpaB was performed with the EMBOSs alignment tool (http://www.ebi.ac.uk/emboss/align). Shown are the percentage amino acid identities to YopB across the entire PopB or IpaB proteins or across the regions encompassing their putative transmembrane domains 1 (TM1) and 2 (TM2) and the coiled-coil domains 1 (CC1) and 2 (CC2). The lengths of matching regions after introduction of gaps to optimize the alignment are shown in parentheses. The '?' symbol highlights a domain that was only predicted when using low-stringency parameter settings. A third CC domain was also identified in the N terminus of IpaB, in a region that displays no obvious similarity with YopB.

Fig. 4. Sequence comparison of the YopB, PopB and IpaB translocator proteins. The conserved architecture of each protein is represented. Predicted coiled-coil regions (CC, dark grey) were identified using the COILS web server (http://www.ch.embnet.org/software/COILS_form.html) and membrane-spanning regions (TM, light grey) were predicted using the TMPRED web server (http://www.ch.embnet.org/software/TMPRED_form.html). A pairwise alignment of YopB with PopB or IpaB was performed with the EMBOSs alignment tool (http://www.ebi.ac.uk/emboss/align). Shown are the percentage amino acid identities to YopB across the entire PopB or IpaB proteins or across the regions encompassing their putative transmembrane domains 1 (TM1) and 2 (TM2) and the coiled-coil domains 1 (CC1) and 2 (CC2). The lengths of matching regions after introduction of gaps to optimize the alignment are shown in parentheses. The '?' symbol highlights a domain that was only predicted when using low-stringency parameter settings. A third CC domain was also identified in the N terminus of IpaB, in a region that displays no obvious similarity with YopB.

expressed from pJEB44 (PopD⁺) (Fig. 3, compare G with A and J), or together with PcrH (pJEB53) (Fig. 3, compare H with A and K), was unable to mediate a YopE-dependent cytotoxic response towards HeLa cells, even after a prolonged infection (7 h, data not shown). To investigate whether this was simply due to a poor translocation efficiency, we took advantage of the hyper-translocation phenotype observed with Yersinia strains defective in YogK (Francis & Wolf-Watz, 1998; Holmström et al., 1997) and utilized the previously characterized ΔyopK yopD double mutant (YPIII/pIB155D) of Yersinia (Francis & Wolf-Watz, 1998). However, even in this background, PopD was unable to restore the YopD-defective phenotype (data not shown). Furthermore, to rule out the possibility that PopD/YopD confers effector substrate specificity during the translocation process, we introduced exoenzyme S (ExoS) under its native promoter (pTS103-Gm) (Bröms et al., 2003a) into YPIII/pIB621 already expressing PopD (pJEB44). ExoS is a potent cytotoxin translocated by the TTSS of P. aeruginosa (Barbieri, 2000), but is also efficiently translocated by surrogate strains of Yersinia spp. (Bröms et al., 2003a; Frithz-Lindsten et al., 1997; Henriksson et al., 2002). Akin to the inability of PopD produced in Yersinia to promote translocation of YopE, we did not observe any evidence of ExoS-dependent cytotoxicity towards HeLa cells infected with YPIII/pIB621(pJEB44, pTS103-Gm) (Fig. 3, compare I with A and F). However, we ensured that the failure to complement the ΔyopD null mutant was not due to a defective experimental set-up, because this mutant trans-complemented with native YopD alone [YPIII/pIB621(pJEB54)] or co-produced with native LcrH [YPIII/pIB621(pJEB91)], respectively, induced a YopE-dependent cytotoxicity on infected HeLa cells, reminiscent of an infection with wild-type Yersinia (Fig. 3, compare J and K with A). Thus, this suggests that key motifs in YopD, which are not conserved in PopD, are essential for formation of a translocation-competent TTSS in Yersinia.

PopD alone or co-produced with PcrH fails to restore normal yop-regulatory control in Y. pseudotuberculosis

Both ΔyopD and ΔlcrH yopD null mutants have lost the ability to maintain regulatory control of Yop synthesis (Francis & Wolf-Watz, 1998; Francis et al., 2000, 2001; Williams & Straley, 1998). This is borne out by the constitutive synthesis of Yop proteins at 37 °C, and in the uncontrolled secretion of LcrV, regardless of the presence (non-inducing Yop synthesis and secretion conditions) or absence (inducing conditions) of calcium in the growth media. This loss of regulatory control is also reflected in bacterial growth restriction upon temperature upshift from 26 to 37 °C, independent of calcium (termed temperature-sensitive, TS) (Bergman et al., 1991; Francis & Wolf-Watz, 1998; Francis et al., 2001; Williams & Straley, 1998). The growth phenotype of wild-type Y. pseudotuberculosis is calcium-dependent (CD), implying that this strain requires calcium for growth at 37 °C and in so doing exhibits a
normal pattern of Yop synthesis (Bergman et al., 1991; Francis & Wolf-Watz, 1998). The regulatory defects associated with loss of YopD and/or LcrH are consistent with the requirement of a YopD–LcrH complex to maintain yop-regulatory control (Anderson et al., 2002; Francis et al., 2001). In view of this, we investigated whether PopD (pJEB44) or PopD and PcrH (pJEB53) could complement the TS phenotype displayed by the DyopD (YPIII/pIB621) or DlcrH yopD (YPIII/pIB621H) deletion mutants, respectively (Table 2). However, PopD produced alone (pJEB44) or in combination with PcrH (pJEB53) could not alter the TS growth phenotype of these Yersinia mutants, even though native YopD (pJEB54) or YopD with LcrH (pJEB91) could readily restore the growth defects from TS to wild-type CD, respectively (Table 2). Furthermore, the inability of P. aeruginosa alleles popD (pJEB44) (Fig. 6, compare lanes g and h with a and b) or popD and pcrH (pJEB53) (Fig. 6, compare lanes z and aa with a and b) to complement the regulatory defects in Yersinia DyopD (YPIII/pIB621) or DlcrH yopD (YPIII/pIB621H) deletion mutants, respectively, was also reflected in constitutive Yop production and the uncontrolled secretion of LcrV at 37 °C regardless of Ca²⁺ levels. In contrast, these same mutants, respectively producing YopD alone (pJEB54) (Fig. 6, compare lanes k and l with a and b) or in combination with LcrH (pJEB91) (Fig. 6, compare lanes dd and ee with a and b), restored yop-regulatory control in that production and secretion of Yop proteins and LcrV was characteristically induced at 37 °C in media devoid of Ca²⁺. While these data may simply relate to genetic differences between YopD/PopD and/or LcrH/PcrH, our interpretation is that the regulatory function of the YopD–LcrH complex might be unique to Yersinia type III secretion.

DISCUSSION

A high degree of genetic conservation characterizes the type III apparatus and associated translocator components of P. aeruginosa and Yersinia (Frank, 1997; Hueck, 1998). No doubt these features contribute to the ability of the pcrGVHpopBD translocation operon of P. aeruginosa to restore Yop-effector translocation into cells infected with 

Fig. 6. Analysis of Yop/Pop synthesis and secretion from Y. pseudotuberculosis strains grown either with (+) or without (−) Ca²⁺. Yop/Pop proteins were separated by SDS-PAGE and identified either by immunoblot analysis (upper panel) using polyclonal rabbit anti-YopH, anti-LcrV, anti-YopD, anti-PopD and anti-YopE antiserum (total protein fraction – a mix of Yop/Pop proteins secreted to the culture medium and contained within intact bacteria) or by Coomassie brilliant blue staining (lower panel) of TCA-precipitated secreted proteins (derived from cleared culture supernatants). Where indicated, IPTG was added at a final concentration of 0.4 mM upon temperature shift. Lanes: a and b, wild-type YPIII/pIB102; c and d, ΔyopD null mutant YPIII/pIB621; e–h, complemented YPIII/pIB621 (pJEB44); i–l, complemented YPIII/pIB621 (pJEB54); m and n, ΔlcrH yopD null mutant YPIII/pIB621H; o–r, complemented YPIII/pIB621H (pJEB44); s–v, complemented YPIII/pIB621H (pJEB54); x–aa, complemented YPIII/pIB621H (pJEB53); bb–ee, complemented YPIII/pIB621H (pJEB91). The single asterisk indicates secreted PopD, while the double asterisk indicates secreted YopD. Molecular masses, shown in parentheses, were deduced from primary sequence.
corresponding deletion mutants of *Yersinia* (Bröms et al., 2003a; Frithz-Lindsten et al., 1998). In this study, we were keen to address the molecular mechanisms of this common virulence strategy using a functional comparison approach. We observed that PopB could complement a YopB-defective mutant as measured by the ability of PopB to facilitate effector translocation in the presence of other *Yersinia* components, but only when co-expressed with native PcrH chaperone. In contrast, production of PopD in *Yersinia* could not overcome the pleiotrophic defects of a Δ*yopD* null mutant despite significant PopD secretion by *Yersinia*, even in the presence of non-native LcrH chaperone. Collectively, these findings highlight the validity of our experimental approach and suggest that YopD-specific amino acids are responsible for mediating type III secretion in *Yersinia*.

The fact that PopD could not cooperate with *Yersinia* components to complement a *Yersinia ΔyopD* null mutant is an exciting observation, especially considering that co-production with *P. aeruginosa* components encoded within the *pcrGVHpopBD* operon was sufficient for complementation (Bröms et al., 2003a; Frithz-Lindsten et al., 1998). This was not due to variable PopD secretion, since relative levels were comparable in each experimental set-up. Furthermore, it was not a result of different origins of PopD, as the PopD sequence of the complementing *pcrGVHpopBD* operon derived from *P. aeruginosa* 388 (Bröms et al., 2003a; Frithz-Lindsten et al., 1998), and the non-complementing *popD* allele from strain PAK (this study), showed no sequence diversity at the protein level (data not shown). In addition, other *P. aeruginosa* translocron components including PcrH (Bröms et al., 2003b), PopB (this study) and, to a lesser extent, PcrV (Bröms et al., 2003a; Holmström et al., 2001; Pettersson et al., 1999) could all individually recognize *Yersinia* components by complementing the translocation defects in the corresponding single mutants of *Yersinia*. Moreover, the ability of PopB, but not IpaB of *Shigella*, to complement a Δ*yopD* null mutant is intriguing considering their proposed structural and functional similarities (Håkansson et al., 1993; Kaniga et al., 1995). We interpret this to imply that additional regions conserved in YopB and PopB, but absent in IpaB, permit this functional conservation of PopB in *Yersinia*. This clearly serves to reinforce the common type III virulence strategy employed by both *P. aeruginosa* and pathogenic *Yersinia* spp. Moreover, our data are suggestive of critical domains within YopD that are necessary for a functional TTSS in *Yersinia*. In view of our data, the recent report suggesting YscF, the needle component of the *Yersinia* secretion apparatus, to be the sole requirement for translocation is paradoxical (Hoiczyk & Blobel, 2001). However, it may indicate the occurrence of a key YscF–YopD interaction that is important for functional type III secretion in *Yersinia*.

In *Yersinia*, dual roles for YopD are known. YopD is essential for both translocation of Yop-effectors and maintenance of the negative control loop governing regulation of type III secretion (Bergman et al., 1991; Francis & Wolf-Watz, 1998; Williams & Straley, 1998). This requires LcrH to ensure pre-secretory stabilization and efficient secretion of YopD (Francis et al., 2000; Wattiau et al., 1994). In addition, a role for LcrH in yop-regulatory control is also envisaged (Anderson et al., 2002; Francis et al., 2001). Interestingly, both *Yersinia* spp. and *P. aeruginosa* TTSSs are responsive to low calcium levels *in vitro* (Frank, 1997; Straley et al., 1993; Sundin et al., 2002) and target cell contact *in vivo* (Pettersson et al., 1996; Rosqvist et al., 1994; Vallis et al., 1999). Therefore, it is significant that even with common inductive responses and high amino acid relatedness, *P. aeruginosa*-derived PopD alone or in combination with PcrH was unable to complement the regulatory defects of Δ*yopD* or Δ*lcrH yopD* null mutants of *Yersinia*. This was not due to the absence of other *P. aeruginosa* translocase components because the Δ*lcrG*Δ*yopD* operon deletion mutant of *Yersinia* complemented with the corresponding *P. aeruginosa* operon did not regain yop-regulatory control either, even though the ability to translocate Yop-effectors was efficiently restored (Bröms et al., 2003a). This regulatory defect is specific to PopD and PcrH, because both PcrG and PcrV of *P. aeruginosa* do complement regulatory deficient Δ*lcrG* (Matson & Nilles, 2002) and Δ*lcrV* (Bröms et al., 2003a; Pettersson et al., 1999) null mutants of *Yersinia*, respectively. Furthermore, this specific defect was not due to the inability to form a chaperone–substrate complex, since a heterologous PopD–LcrH complex has been observed (Allmond et al., 2003; Bröms et al., 2003b), which is consistent with the ability of LcrH to promote stability and secretion of PopD in *Yersinia* (this study). Clearly, therefore, responsiveness to common environmental cues belies the development of independent regulatory networks in *Yersinia* and *P. aeruginosa*. Apparently, the PopD–PcrH complex in *P. aeruginosa* does not mimic the essential regulatory function of the YopD–LcrH complex in *Yersinia*. This is supported by the fact that Δ*yopD* (Sundin et al., 2002) and Δ*pcrH* (Bröms et al., 2003b) deletion mutants of *P. aeruginosa* do not display any detectable regulatory phenotype. With respect to the regulatory role of LcrH, an alignment with PcrH does not clearly define a unique, putative regulatory domain(s) in LcrH (data not shown). Nonetheless, the termini of these two proteins are less well conserved than the internal region, which might be indicative of a unique role in their native context. This is currently under investigation.

Interestingly, the function of the LcrQ repressive element involved in *Yersinia* Yop production (Rimpilaäinen et al., 1992) seemingly requires a YopD–LcrH complex to respond to *in vitro* calcium levels or *in vivo* target cell contact (Cambronne & Schneewind, 2002; Williams & Straley, 1998). However, an LcrQ homologue is absent in the *P. aeruginosa* genome (Stover et al., 2000), perhaps this necessitates the need for a different regulatory mechanism in *P. aeruginosa*. In this context, it is interesting that the *P. aeruginosa* genome sequencing project has revealed a high proportion of the genome that is dedicated to encoding regulatory factors (Stover et al., 2000), some of which likely
relieve this bacterium of the requirement for a PopD–PcrH complex involved in regulation of the TTSS.

In vitro studies have implicated YopD in complexes with structural components including YopB (Hartland & Robins-Browne, 1998; Neyt & Cornelis, 1999b), LcrV (Sarker et al., 1998) and TyeA (Cheng & Schneewind, 2000; Iriarte et al., 1998), in addition to the customized chaperone LcrH (Francis et al., 2000; Wattiau et al., 1994) and the translocated YopE effector (Hartland & Robins-Browne, 1998). Presumably, these interactions form the nucleus of functional translocation in Yersinia, such that PopD is deficient in one or more of these interactions. If YopD binding to Yop-effectors is critical for substrate recognition by the translocase apparatus, PopD might be precluded from binding to Yersinia effectors. However, the full P. aeruginosa translocase operon can restore translocation in a complete operon deletion mutant of P. aeruginosa (Bröms et al., 2003a), suggesting that PopD does possess the capacity to recognize Yersinia effectors when in the right molecular context. It follows that the participation of PopD in the translocator complex composed of native Yersinia components not only failed to translocate Yersinia effectors, but also P. aeruginosa ExoS. Therefore, our data clearly indicate that a specialized interaction of YopD with one or more members of the Yersinia translocase apparatus establishes functional translocation in this bacterium. Using this experimental set-up, a molecular approach is now in progress to determine this key YopD/PopD-dependent interaction. In this context, a YopB—YopD complex apparently contributes to pore formation in eukaryotic plasma membranes (Håkansson et al., 1996; Neyt & Cornelis, 1999a; Tardy et al., 1999) that also involves LcrV (Bröms et al., 2003a; Holmström et al., 2001). It is assumed that this complex incorporates a translocation pore through which anti-host Yop effectors traverse to gain entry into the eukaryotic cell. We envisage that the generation of PopD/YopD hybrid proteins or functional intergenic PopD suppressors would identify the key molecular interactions and allow functional domains to be mapped. These approaches have proven successful in analysing comparative functional domains of P. aeruginosa PcrV and Y. pseudotuberculosis LcrV (Holmström et al., 2001) and the delineation of the YopD–LcrH complex (Francis et al., 2000, 2001).

Noteworthy is the prediction of structural regions within YopD that are likely to play a key role in Yersinia type III secretion. Such a role is known for the C-terminal amphipathic domain (Tengel et al., 2002), which is essential for Yop effector translocation (Francis & Wolf-Watz, 1998). Interestingly, a helical wheel projection (ANTHEPROT version 3.2; G. Deleage, France) over the equivalent region in PopD only predicted an α-helix with marginal amphipathic properties (data not shown). Furthermore, the coiled-coil domain of YopD, a structure common to many proteins secreted by TTSSs (Delahay & Frankel, 2002; Pallen et al., 1997), is not predicted in PopD using the COILS web server (http://www.ch.embnet.org/software/COILS_form.html) even with low stringent parameter settings (data not shown). As these structural differences might prevent PopD from recognizing Yersinia type III components, they represent the focus of our ongoing studies.

In summary, we have confirmed a functional conservation of the translocase operons from P. aeruginosa and Yersinia. Like PcrV (Bröms et al., 2003a; Pettersson et al., 1999) and PcrH (Bröms et al., 2003b), PopB of P. aeruginosa can adequately interact with members of the translocase apparatus of Yersinia and allow effector translocation. However, the action of PopD in P. aeruginosa and YopD in Yersinia is different. The former was unable to restore yop-regulatory control or effector translocation in YopD-defective Yersinia. This study points to key regions within YopD, but not PopD, which are required for efficient type III translocation during Yersinia infections. We are currently exploiting the compatibility between the P. aeruginosa and Yersinia systems to identify the important YopD-dependent events that elevate type III secretion into a powerful virulence strategy during Yersinia infections.

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