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TnphoA mutants of *Providencia alcalifaciens* with altered invasiveness of HEP-2 cells

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Recent studies have shown that *Providencia alcalifaciens* is a diarrhoeal pathogen. It may cause diarrhoea by an invasive mechanism, as it invades cultured mammalian cells *in vitro* and intestinal epithelial cells of experimentally inoculated rabbits *in vivo*. To locate the gene(s) involved in invasion, TnphoA mutants of a diarrhoeal isolate of *P. alcalifaciens* were generated. Compared with the parent strain, these mutants exhibited negligible invasion and actin condensation in HEP-2 cells. TnphoA insertion was located in fragments of 4.9 kb and 11.1 kb of the bacterial chromosome by Southern blot. These mutants did not secrete a 28-kDa protein, which may be involved in invasion. It should be possible now to study the gene(s) involved in invasion of *P. alcalifaciens* with these mutants. This investigation is another example of the usefulness of TnphoA mutagenesis in the study of bacterial virulence genes.

Introduction

There are five species in the genus *Providencia* [1]. At least one species, *P. alcalifaciens*, has been implicated in diarrhoea. The evidence for an aetiological role of *P. alcalifaciens* in diarrhoea has come from studies on travellers' diarrhoea [2], case-control studies of diarrhoea [3] and pathogenicity studies of isolates [4]. The pathogenicity studies have indicated that the organism may cause diarrhoea by an invasive mechanism [4–7]. The bacterial isolates invade cultured mammalian cells *in vitro* [4–7], induce fluid accumulation in the ligated rabbit ileal loop [4] and induce diarrhoea in the removable intestinal tie adult rabbit diarrhoea (RI-TARD) model [4]. Electron microscopy studies of interaction of the bacterium with rabbit ileal mucosa suggested that there are two modes of invasion of enterocytes: directly through the apical surface and laterally through the intercellular tight junction [5]. Invasion of epithelial cells *in vitro* is associated with condensation of actin filaments, and both invasion and actin condensation can be inhibited by cytochalasin D,

an inhibitor of microfilament formation [6]. Even though some isolates of *P. alcalifaciens* carry plasmids, isolates with and without plasmids were found to be equally invasive, indicating that chromosomal genes, not plasmid genes, are involved in invasion [8]. Also, no homologous sequence corresponding to virulence genes of invasive bacteria such as *Shigella* spp., *Yersinia enterocolitica* and *Y. pseudotuberculosis* could be found [8]. In many bacteria, genes encoding surface-exposed or secreted products can be identified by TnphoA mutagenesis. The mutants are detected by screening for alkaline phosphatase activity on an indicator medium [9, 10]. In many invasive bacteria, including *Shigella* spp., secreted products are involved in invasiveness [11]. It was reasoned that the gene(s) associated with invasion in *P. alcalifaciens* encodes surface-exposed or secreted products. Therefore, TnphoA mutants of *P. alcalifaciens* were generated and studied to locate the gene(s) involved in invasion.

Materials and methods

Bacterial strains and media

P. alcalifaciens 2939/90, a wild-type, spontaneous tetracycline-resistant strain of *P. alcalifaciens* isolated from the rectal swab of a patient with diarrhoea, was

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used as the recipient strain to generate mutants [4]. *Escherichia coli* SM10 λ *pir* containing plasmid pRT733 was used as the donor for *TnphoA*. The plasmid pRT733 contains *TnphoA* in a suicide vector [10]. *Shigella flexneri* 2a 611R and *E. coli* K-12 were used as positive and negative controls in invasion assays [4]. The media used for growing bacteria were Luria broth (LB) or Luria agar (LA). Whenever incorporated in media, kanamycin was used at 30 mg/L, tetracycline at 15 mg/L and gentamicin at 100 mg/L.

TnphoA mutagenesis

TnphoA mutagenesis of *P. alcalifaciens* 2939/90 was performed by conjugation as described previously [10]. The donor strain of *E. coli* SM 10 λ *pir* was grown in LB with kanamycin and the recipient strain of *P. alcalifaciens* 2939/90 was grown in LB with tetracycline on an orbital shaker (Innova 4300, New Brunswick Scientific, NJ, USA) at 120 rpm at 37°C for 20 h. The bacteria were harvested by centrifugation, washed once in phosphate-buffered saline (PBS, pH 7.2) and resuspended in LB to a final concentration of 10^9 cfu/ml each. The donor and recipient strains were mixed at a ratio of 10:1 and incubated with vigorous shaking (120 rpm) at 37°C for 4 h. Then 200- μ l samples of the mating mixed culture were spread on LA plates containing kanamycin, tetracycline, dextrose (0.2%) and the chromogenic substrate, 5-bromo, 4-chloro, 3-indolyl phosphate (XP; Sigma) 100 μ g/ml and incubated at 37°C for 24–48 h. Transconjugants with *TnphoA* inserted in a functional gene (as indicated by deep blue colonies) were selected. After checking the purity of the colonies by streaking on fresh XP plates, the colonies were stored in LB containing kanamycin and tetracycline and glycerol 15% at –70°C for further study.

Assay for motility

The motility of wild-type *P. alcalifaciens* and its *TnphoA* mutants was determined by the hanging-drop method with a 6-h LB culture incubated at 37°C and also after incubation for 18 h at 37°C of an inoculated soft nutrient agar tube.

HEp-2 cell invasion assay

The HEp-2 cell invasion assay was performed as described by Albert *et al.* [4]. HEp-2 cell monolayers in 24-well cell culture plates (Corning, NY, USA) grown in minimum essential medium (MEM) containing fetal bovine serum (Gibco BRL, Grand Island, NY, USA) 10% were washed twice in MEM and infected with wild-type *P. alcalifaciens* and its *TnphoA* mutants (1×10^7 cfu each to give a multiplicity of infection of 100). *S. flexneri* 2a 611 R and *E. coli* K-12 were used as positive and negative controls respectively in each experiment. After a 2-h infection period, the monolayer

was incubated for 1 h in MEM containing gentamicin to kill extracellular bacteria (the parent strain and mutants were susceptible to gentamicin). Various dilutions of lysed monolayer were plated on LA plates for enumeration of intracellular *S. flexneri* 2a, *E. coli* K-12 and wild-type *P. alcalifaciens*. For *TnphoA* mutants, the LA plate contained both kanamycin and tetracycline. The experiments were performed three times in duplicate.

HEp-2 cell-associated organisms

To determine cell-associated organisms, the assay was performed as described above for invasion. However, after the 2-h infection period, the monolayer was washed three times with MEM, lysed and varying dilutions were plated for enumeration of bacteria.

Fluorescent actin staining (FAS) test

The ability of bacteria to condense actin was tested in a HEp-2 cell assay as described previously [4]. The degree of condensation was scored on a scale of 0–4+ [6]. *S. flexneri* 2a 611 R and *E. coli* K-12 were used as positive and negative controls, respectively, in each experiment. The experiments were performed three times in duplicate. The condensation produced by *S. flexneri* was assigned a value of 4+.

Southern blot hybridisation

The chromosomal DNA was extracted from the wild-type *P. alcalifaciens* and its mutants by the standard phenol-chloroform method [12]. It was then digested with *Bam*HI enzyme (Gibco BRL) according to manufacturer's protocol. This enzyme does not cut within the *TnphoA* gene. The digested DNA (30 μ l) was separated on an agarose (Type II: medium EEO; Sigma) 0.8% gel and transferred to a nylon membrane (Hybond TM-N⁺, YA0147; Amersham, IL, USA) with a vacuum blotter (BioRad, CA, USA). The DNA material was fixed to the membrane by exposure to UV light. A portion of kanamycin resistance gene was used as a probe to locate *TnphoA* insertion in the chromosome. The kanamycin gene was amplified from recombinant plasmid pRT773 with primers *kanI* (5' AGC TGG GGC GAG CTC TGG TAA GGT 3') and *kanII* (5' AAA GGG AAT AAG GTC GAC ACG GAA 3'). The amplified product was labelled by digoxigenin-11-dUTP by the random primer extension method with a DIG DNA labelling and detection kit (Boehringer-Mannheim, Mannheim, Germany). The hybridisation and detection were performed according to the manufacturer's instructions.

Antiserum to *P. alcalifaciens*

Rabbit polyclonal antiserum to the wild-type *P. alcalifaciens* was produced by a previously described protocol [12]. Briefly, the culture was grown in LB at

37°C for 20 h on an orbital shaker (Innova 4300, New Brunswick Scientific). Antiserum was produced in an adult New Zealand White rabbit. The rabbit was given four intravenous injections at weekly intervals, with a booster injection 3 weeks after the last injection. The amount of culture administered each time was 500 µl. The rabbit was exsanguinated 7 days after the last dose.

SDS-PAGE and immunoblotting

Single colonies of the parent *P. alcalifaciens* were grown in 5 ml of LB and those of mutants in 5 ml of LB with kanamycin and tetracycline. The cells were pelleted by centrifugation and the protein in the supernate was precipitated with ammonium sulphate 60% w/v. The proteins from lysed whole-cell pellet and supernate were analysed separately. Samples containing 10 µg total protein were separated on 10% SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane for immunoblotting [13]. The membrane was exposed to rabbit polyclonal antiserum to *P. alcalifaciens* (1 in 500 dilution) and then to affinity-purified horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin G (1 in 500 dilution) (Sigma). Bound conjugate was detected with the substrate containing ice-cold methanol 16%, 4-chloro, 1-naphthol 0.05% and H₂O₂ 0.025% in Tris-buffered saline.

Results

Generation of *TnphoA* transconjugants

Two separate experiments yielded 59 mutants from *P. alcalifaciens* 2939/9. The frequency of transfer of *TnphoA* varied from 5.0×10^{-9} to 5.0×10^{-8} .

Motility

Like the wild-type strain of *P. alcalifaciens*, all the three mutants were motile by both hanging-drop method and soft agar method.

HEp-2 cell association, invasion and actin condensation by bacteria

Of the 59 mutants, 13 showed reduced invasion compared with the parent strain on initial screening. However, on further screening, only four mutants (nos 23, 47, 63 and 78) showed consistently reduced invasion. These mutants only were studied further. The cell association, invasion and actin condensation are shown in Table 1. The parent and mutant strains showed similar levels of cell association. However, compared with the parent strain, the mutants showed negligible invasion. There was a 33–111-fold reduction in invasion. Whereas the parent strain showed strong actin condensation, the mutants showed doubtful condensation.

Southern blot hybridisation

The *TnphoA* insertion in the four invasion-deficient mutants was located by Southern blot hybridisation. There appeared to be a single insertion in all four mutants. The transposon was located in a fragment of 4.9 kb in mutant nos 23 and 47, and in a fragment of 11.1 kb in mutant nos 63 and 78 (Fig. 1).

Immunoblot analysis of proteins

To investigate whether *TnphoA* insertion had disrupted the expression of any functional gene encoding secreted fusion protein, the protein profiles of the parent and the four mutants (nos 23, 47, 63 and 78) were studied by immunoblotting. This showed that all four mutants failed to secrete a 28-kDa protein. Fig. 2 shows the presence of the 28-kDa protein in the parent strain and mutant no. 51 (a mutant that was not invasion-deficient) and its absence in mutant nos 23, 47, and 63. No differences between the parent strain and the mutants could be detected in the immunoblot when the lysed whole-cell pellet was analysed (data not shown).

Discussion

TnphoA insertion mutagenesis has been used successfully to characterise genes encoding surface-exposed or

Table 1. Cell-association, invasion and actin condensation of *P. alcalifaciens* and its mutants with HEp-2 cells*

Strain	Bacteria (cfu)/ml		Intracellular bacteria relative to inoculum (%)	Actin condensation
	Cell-associated	Intracellular		
<i>P. alcalifaciens</i> (2939/90)	2.0×10^5	1.0×10^4	0.1	3+
Mutant 23	2.5×10^5	3.6×10^2	0.003	±
Mutant 47	2.9×10^5	9.0×10^1	0.0009	±
Mutant 63	3.0×10^5	1.8×10^2	0.001	±
Mutant 78	3.2×10^5	2.9×10^2	0.002	±
<i>S. flexneri</i> 2a (611R)	ND	4.0×10^5	4.00	4+
<i>E. coli</i> K-12	ND	0	0	–

ND, not done.

*Values are averages of duplicate samples and represent data from one of three experiments. The inoculum was 1.0×10^7 for all bacteria.

Fig. 1. Southern blot hybridisation of *P. alcalifaciens* 2939/90 and its *TnphoA* mutants. Chromosomal DNA from all isolates was digested with *Bam*HI and probed with digoxigenin-labelled kanamycin resistance gene. Lane 1, amplified kanamycin resistance gene (positive control); 2, *P. alcalifaciens* 2939/90; 3, mutant 23; 4, mutant 47; 5, mutant 63; 6, mutant 78.

Fig. 2. Immunoblot analysis of secreted proteins of *P. alcalifaciens* 2939/90 and its *TnphoA* mutants. The proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with rabbit polyclonal antibody to *P. alcalifaciens* 2939/90 and enzyme-conjugated anti-rabbit immunoglobulin. Lane 1, *P. alcalifaciens* 2939/90; 2–5, *TnphoA* mutants 23, 47, 51 and 63, respectively.

secreted proteins in many bacteria [14–16]. In the present study, four mutants of PA that had negligible invasiveness and actin condensation in HEP-2 cells compared with the parent strain were generated by the same strategy. The reduction observed in these properties could not be attributed to differences in motility and cell association between the parent strain and the mutants, as these properties were not altered in the mutants compared to the parent strain. Single *TnphoA* insertions were located in fragments of two different sizes in these mutants; this suggested that more than one locus is involved in invasion. All four mutants failed to secrete a 28-kDa protein. In many bacteria, including *Salmonella* spp. [17] and *Shigella* spp. [18], secreted proteins are involved in invasion of mammalian cells. It is likely that the 28-kDa secreted protein may also be involved in the invasiveness of

P. alcalifaciens. When the lysed whole-cell pellet was analysed in immunoblot, no difference in protein profile was observed between the parent strain and the mutants. This suggested that other proteins may not be involved in the invasion process. It should be now possible to perform molecular characterisation of gene(s) involved in invasiveness of *P. alcalifaciens* with the help of these mutants. Some further studies involve sequencing the *TnphoA* insertion site, comparison of sequence with sequence data in the literature, cloning the gene, complementation of mutants with the cloned gene and expression of the cloned gene. The present study has added *P. alcalifaciens* to the list of pathogenic bacteria whose virulence determinants can be studied by *TnphoA* mutagenesis.

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