Edwardsiella tarda mutants defective in siderophore production, motility, serum resistance and catalase activity

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Edwardsiella tarda is a Gram-negative bacterium that causes a systemic infection, edwardsiellosis, in fish. The virulence factors of this pathogen and its genetic determinants have not been systematically examined. In this study, TnphoA transposon mutagenesis was used to construct a library of 440 alkaline phosphatase (PhoA⁺) fusion mutants from a total of 400 000 transconjugants derived from Ed. tarda PPD130/91. This library included genes for secreted and membrane-associated proteins normally involved in virulence. The library was screened for four virulence factors: siderophore production, motility, serum resistance and catalase production. Eight mutants deficient in one or more of these phenotypes were grouped into four classes. They were further characterized for their stimulation of reactive oxygen intermediate production by fish phagocytes, for their adhesion to and internalization into EPC (epithelioma papillosum of carp) cells, and for attenuation of virulence in blue gourami. Mutants 2A and 34 were highly attenuated in fish, with LD$_{50}$ values about 10 times higher than for the wild-type. These strains had mutations in the genes encoding arylsulfate sulfotransferase (mutant 2A) and a catalase precursor protein (mutant 34). One hyperinvasive/adhesive mutant and four pst mutants that were pleiotropic and slightly attenuated in fish were also isolated.

Keywords: fish pathogen, transposon mutagenesis, virulence genes

INTRODUCTION

Edwardsiella tarda is the causative agent of edwardsiella septicaemia, which affects several commercially important feral and farmed fish species and is widely distributed in the aquatic environment (Kusuda & Salati, 1993; Thune *et al*., 1993; Rashid *et al*., 1994). The principal concerns are the severe lethality of the disease in intensive culture systems and the pathogen's broad host range, which includes humans (Plumb, 1993). Little information is available regarding the virulence and genetic make-up of this pathogen. Virulence factors such as adhesion and invasion of epithelial cells (Strauss *et al*., 1997; Ling *et al*., 2000), anti-phagocyte-mediated killing (Ainsworth & Dexiang, 1990), production of

haemolysin and other toxins (Janda *et al*., 1991a; Suprapto *et al*., 1996), and serum resistance (Janda *et al*., 1991b) have been implicated in the pathogenesis of *Ed. tarda*. Although genetic determinants involved in haemolysin production have been elucidated (Chen *et al*., 1996; Hirono *et al*., 1997), other virulence genes remain largely unknown.

A more systematic approach to the study of *Ed. tarda* pathogenesis is needed to identify and characterize the virulence factors and their mechanisms in mediating diseases in fish and other animals. To understand the mode of pathogenesis, it is imperative to study the virulence factors that predispose a stressed population of fish to the disease. Among these, flagella-mediated motility is believed to be crucial in the early stages of pathogenicity, which involve stable attachment and mucosal colonization (Otteman & Miller, 1997). The persistence of bacterial infection depends on the pathogen's ability to acquire nutrients such as iron from the host (Guerinot, 1994; Finlay & Falkow, 1997; Payne,

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1993). Most pathogens secrete high-affinity iron chelators (siderophores) and transport siderophore–Fe^{3+} complexes into the cell interior (Mietzner & Morse, 1994; Ferguson *et al*., 1998). Pathogens may also produce catalase against phagosomal reactive oxygen intermediates (ROI) such as H_2O_2 (Miller & Britigan, 1997) and other factors to evade non-specific defence systems of the host like the bactericidal effect of serum (Yano, 1996).

Transposon mutagenesis can be used to examine various aspects of bacterial virulence factors and their genetic determinants (Lodge *et al*., 1995; Berg & Berg, 1996; Bina *et al*., 1997). Attenuated mutants created by the insertion of transposons in genes associated with virulence can help to elucidate host–pathogen interactions and invasion pathways. Most virulence factors are either on the bacterial surface or secreted such that they can interact with host components (Finlay, 1996). In this study we therefore chose a transposon, Tn*phoA*, to identify potential virulence genes involved in the production of secretory or membrane proteins. We isolated 440 PhoA$^+$ fusion mutants of *Ed. tarda* PPD130/91 employing Tn*phoA* mutagenesis. Disruption of genes involved in iron transport, serum survival, motility and catalase could attenuate the pathogen, impairing its ability to cause infection in fish. Our results suggest the possible correlation of these factors with the virulence of the pathogen; the information elucidated will enrich the existing knowledge on *Ed. tarda* pathogenesis.

METHODS

Bacterial strains and growth conditions. *Edwardsiella tarda* PPD130/91, a virulent strain isolated from diseased fish (Ling *et al*., 2000) was used as the recipient for transposon mutagenesis. *Escherichia coli* SM10 λpir(pJM703.1::Tn*phoA*) (Manoil & Beckwith, 1985) was used as the donor for Tn*phoA* in the mating experiment. The identity of *Ed. tarda* PPD130/91 was tested as reported previously (Ling *et al*., 2000). Cultures were routinely grown in tryptic soy broth (TSB, Difco) or on tryptic soy agar (TSA, Difco) at 25 °C. Stock cultures were maintained at −80 °C in TSB containing 25 % (v/v) glycerol. When required, the antibiotics (Sigma) ampicillin (Amp), neomycin (Neo), colistin (Col), gentamicin (Gm) and kanamycin (Kan) were added at final concentrations of 50, 50, 12·5, 100 and 50 µg ml^{-1}, respectively. Growth under phosphate-limiting conditions was examined by culturing the bacterial cells in a defined minimal medium (modified DMM) (Collins & Thune, 1996) with the phosphate salts substituted by 3 µM Na_2HPO_4 and the pH adjusted to 7·0 with 30 mM HEPES.

Cell culture and media. *Epithelioma papillosum* of carp, *Cyprinus carpio* (EPC) cells (Wolf & Mann, 1980) were grown in minimal essential medium (MEM) with Hanks' balanced salts solution (HBSS) (Sigma), 10 mM HEPES (pH 7·3), 2 mM glutamine, 0·23 % $NaHCO_3$ and 10 % (v/v) heat-inactivated fetal bovine serum at 25 °C in a 5 % (v/v) CO_2 atmosphere. Cells were grown in 75 ml flasks and split at least once a week by trypsin/EDTA treatment with dilution at 1:10 in fresh media. All tissue culture reagents were obtained from Gibco-BRL.

Transposon mutagenesis. *Ed. tarda* (recipient; Col^r) and *E. coli* (donor; Amp^r Neo^r) cultures were grown statically in TSB at 25 °C and 37 °C, respectively. Conjugative transfer of the suicide plasmid pJM703.1::Tn*phoA* was performed by plate mating. Briefly, the bacterial cell ratio of *E. coli* to *Ed. tarda* PPD130/91 was adjusted to 4:1, with a total of about 10^7 c.f.u. of both donor and recipient during mating. After 24 h of mating at 25 °C on TSA plates, the cells were harvested and resuspended in 3 ml phosphate-buffered saline (PBS) (123·2 mM NaCl, 10·4 mM Na_2HPO_4 and 3·2 mM KH_2PO_4, pH 7·2). Appropriate dilutions were plated on TSA supplemented with neomycin, colistin and 5-bromo-3-chloro-3-indolyl phosphate (XP) (Sigma) (40 µg ml^{-1}) (TSANCX) to select for transconjugants of *Ed. tarda*. Blue PhoA$^+$ fusion clones were purified by streaking on TSANCX plates.

Detection of mutants defective in motility, siderophore production and catalase activity. Swarming motility was assayed according to Janda *et al*. (1991b). Fresh bacterial culture was spotted on 0·4 % motility agar. Mutants were considered non-motile if diffused growth around the colony was not observed. Modified M9 minimal medium (Collins & Thune, 1996) supplemented with chrome azurol S (CAS) and 1·5 % agar was used for checking siderophore production according to Neilands (1994). An orange halo around the bacterial colony indicated siderophore production. The results of both these tests were scored after 48 h incubation at 25 °C. To assay catalase activity, a drop of 3 % H_2O_2 solution was added to fresh bacterial colonies on TSA plates. Brisk effervescence was associated with the breakdown of H_2O_2 by endogenous catalase (Hertel *et al*., 1998). Mutants that produced less catalase generated significantly less effervescence than the wild-type.

H_2O_2 inhibition zone test. *Ed. tarda* strains were grown overnight at 25 °C in TSB, and harvested by centrifugation. After washing, the cells were resuspended in fresh TSB at OD_{540} 0·5, and 2 ml of the cell suspension was added into 18 ml top-agar medium, containing TSB and 1 % agar at 50 °C. The top agar was immediately plated onto a Petri dish. After solidification, sterile Whatman 3MM disks (0·6 cm diameter) containing 10 µl 2 mM, 20 mM or 200 mM H_2O_2 were placed on the surface. Zones of inhibition were visualized after incubation overnight at 25 °C (Xu & Pan, 2000).

Survival assay in blue gourami serum. Blood was obtained from naive blue gourami, *Trichogaster trichopterus* (Pallas), and serum was separated from the clot by centrifugation at 4 °C. Bacteria grown in TSB at 25 °C were collected by centrifugation and washed three times in PBS. The bacterial suspension was then mixed with fresh gourami serum to give a final serum concentration of 50 % and the bacterial count was adjusted to approximately 5×10^7 c.f.u. ml^{-1}. Tubes containing bacteria and serum were incubated at 25 °C and 0·1 ml samples were removed after 1 h for serial dilutions and plate counts on TSA. Serum survival ability was calculated by dividing the number of viable bacteria after the serum treatment by the number of viable bacteria before treatment (Wang *et al*., 1998). A value >1 was scored as serum-resistant, while a value <1 indicated serum sensitivity. The data were obtained from three independent experiments.

Superoxide anion assay. Phagocytes were isolated from the head kidney of naive blue gourami and purified according to Secombes (1990). Briefly, head kidney tissue was macerated and filtered through a stainless-steel mesh to produce a cell suspension in L-15 medium (Sigma). Following separation on a 34–51 % (v/v) Percoll (Sigma) gradient, the cells lying in the 34–51 % interface were harvested, washed and resuspended in

L-15 with 5 % (v/v) heat-inactivated fetal calf serum (Sigma). The cells were counted and approximately 0.5×10^6 cells were seeded into each well of a 96-well tissue culture plate (Falcon) followed by incubation for 2 h at 25 °C in a 5 % (v/v) CO_2 atmosphere. The wells seeded with phagocytes were then inoculated with bacteria at a 1:1 cell ratio and incubated for 30 min at 25 °C. Then 100 µl of 1 mg ml^{-1} nitro blue tetrazolium (Sigma) was added into each well and the cells were further incubated at 25 °C for 30 min in a 5 % (v/v) CO_2 atmosphere. The reaction was arrested with 100 % methanol followed by a single wash with 70 % methanol. After drying the plate for 1 min, 120 µl 2 M potassium hydroxide and 140 µl dimethyl sulfoxide (Sigma) were added. The A_{630} was measured using a microplate reader (BIO-TEK Instruments); the values presented, a measure of ROI production, are means ± SEM of quadruplicate wells from one of the three independent experiments.

Adhesion and internalization assays. These assays were performed as described previously with minor modifications (Tan *et al.*, 1998; Wang & Leung, 2000). Briefly, monolayers of EPC cells were grown for 72 h in 24-well tissue culture plates to 100 % confluence. The cells were then washed with MEM and incubated at 25 °C for 30 min before inoculation of bacteria. The inoculated plates were centrifuged for 5 min at 800 *g* at 4 °C and then incubated for a further 30 min at 25 °C. To measure the number of bacteria adhering to the monolayers, the plates were washed six times with HBSS, the EPC cells lysed with 1 % (v/v) Triton X-100 in PBS, and then bacterial numbers quantified by plate counting (Elsinghorst, 1994). To measure internalization, monolayers were washed twice with HBSS, then incubated for an additional 2 h in tissue culture medium containing 100 µg gentamicin ml^{-1} (Sigma) to kill extracellular bacteria. After incubation, the monolayers were washed twice with HBSS, the EPC cells lysed with 1 % Triton X-100 in PBS and bacterial numbers quantified by plate counting. The adhesion and internalization rates were calculated from the mean of at least two wells in quadruplicate experiments.

DNA manipulations and Southern analysis. Bacterial genomic DNA was extracted according to the manuals of the QIAGEN Genomic DNA Purification Kit and the BIO 101 Genomic DNA Kit. Plasmid DNA was extracted using QIAGEN columns. Restriction endonuclease digestion was accomplished by standard methods (Sambrook *et al.*, 1989). Southern blot analysis was carried out to characterize the transposon mutants of *Ed. tarda* PPD130/91 using the BluGene Non-Radioactive Nucleic Acid Detection System (Gibco-BRL). Transfer of DNA to nylon membrane (GeneScreen, NEN Research Products) and hybridization conditions were in accordance with standard methods (Sambrook *et al.*, 1989). Genomic DNA from *Ed. tarda* 130/91 and its mutants was digested with *Eco*RV, hybridized with *Hin*dIII-digested 14-dATP biotinylated (BioNick Labelling System, Gibco-BRL) pJM703.1 plasmid probe, and then visualized with strepta-vidin–alkaline phosphatase conjugate (BluGene Nonradioactive Nucleic Acid Detection System, Gibco-BRL).

Characterization of transposon insertion sites and DNA sequencing. Genomic DNA of the mutants was digested with *Eco*RV, which did not cut the transposon. Adaptors from the PCR-Select Bacterial Genome Subtraction Kit (Clontech) were then ligated to the *Ed. tarda* DNA fragments, by overnight incubation at 14 °C. PCR was performed using *pfu* Turbo polymerase (Stratagene) with the following cycle parameters: 30 cycles of 1.0 min at 94 °C, 30 s at 58 °C, 30 s at 72 °C and a final extension of 10 min at 72 °C. Primer 1 (Clontech) specific to the adaptor and forward (5′-GCACGATGAAGA-GCAGAAGT-3′) and reverse (5′-GGCATAATTACGTGC-GGCAGT-3′) primers specific to Tn*phoA*, synthesized by Gibco-BRL, were used for amplification. The PCR product was cloned into pT-Adv (Clontech) or pGEM-T Easy vector (Amp^r) (Promega), transformed into *E. coli* Top 10F′ competent cells (Clontech) and spread on LB agar plates containing ampicillin, 40 µl 100 mM IPTG and 40 µl X-Gal (40 mg ml^{-1}) for blue–white screening of cloned DNA. Alternatively, fragments of mutant genomic DNA flanking the transposon obtained by complete digestion with *Bam*HI were cloned into pBluescript SK$^+$ (Amp^r) vector cut with the same enzyme and transformed into *E. coli* TOP10F′ cells as described above. Transformants bearing Tn*phoA* and flanking *Ed. tarda* chromosomal sequences were selected by their ability to grow on LB agar containing ampicillin and neomycin.

DNA sequencing was carried out on an Applied Biosystems PRISM 377 automated DNA sequencer by the dye termination method. The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used. The sequences were edited by using the manufacturer's software. Sequence assembly and further editing were done with DNASIS DNA analysis software (Hitachi Software). TBLASTN, TBLASTX and FASTA sequence homology analyses were performed by using the National Centre for Biotechnology Information BLAST network service.

LD$_{50}$ determinations. Naïve blue gourami weighing approximately 14 g each were purchased from commercial fish farms and acclimatized for 3 weeks. Three groups of 10 fish were injected intramuscularly or intraperitoneally with 0.1 ml PBS-washed bacterial cells adjusted to the required concentrations. A control group of fish each received 0.1 ml PBS. Fish were monitored for mortalities for 14 d and LD$_{50}$ values were calculated by the method of Reed & Muench (1938).

Statistical analysis. Data obtained from adhesion and internalization, serum survival and superoxide anion production assays are expressed as means ± SEM. The data were analysed using one-way ANOVA and a Duncan multiple range test (SAS software, SAS Institute). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Transposon mutagenesis

Approximately 400 000 Neo^r Col^r transconjugants were obtained at a frequency of 7.0×10^{-3} from plate mating, with a spontaneous mutation frequency of $< 4.0 \times 10^{-8}$ for *Ed. tarda* and $< 5.5 \times 10^{-7}$ for *E. coli*. About 0.11 % (440) of the transconjugants in the transposon library expressed active PhoA$^+$ fusion proteins, as determined by the formation of blue colonies on TSANCX plates.

Screening of *Ed. tarda* PPD130/91 mutants for four virulence factors

The putative virulence factors expressed by *Ed. tarda* PPD130/91 include siderophore and catalase production, motility and serum resistance. The 440 PhoA$^+$ transposon mutants derived from this strain were screened to isolate mutants deficient in one or more of

Table 1. General characteristics of the four classes of Tn*phoA* mutants

Class (and no. of mutants)	Siderophore*	Motility†	Catalase‡	Serum survival§
I (1)	$\pm$	+	+	+
IIa and IIb (5)	+	$\pm$/+	+	−
III (1)	+	−	$\pm$	+
IV (1)	+	+	$\pm$	+
130/91 (wt)	+	+	+	+

*Amounts of siderophore produced ranged from low ($\pm$) to high (+) as determined by the size of the orange halo around the colony on CAS agar.

†Motility is scored as '+' (diameter of swarm colony >10 mm), '$\pm$' (diameter of swarm colony 5–10 mm), or '−' (diameter of swarm colony <5 mm).

‡Effervescence (indicating breakdown of H_2O_2 by catalase) in the presence of 3% H_2O_2 is indicated by '+'; '$\pm$' indicates less effervescence.

§Viability in the presence of 50% blue gourami serum over a period of 1 h: +, resistant to serum; −, sensitive to serum.

Table 2. Characterization of mutants of *Ed. tarda* PPD130/91

Within each column, values followed by different superscript letters are significantly different ($P < 0.05$).

Mutant (and class)	ROI production*	Serum assay†	Adhesion (%)‡	Internalization (%)§	Growth in phosphate-limiting medium‖	LD$_{50}$¶ i.m.	i.p.
2A (I)	0.09 ± 0.01^a	$1.54 \pm 0.13^{a,b}$	$4.90 \pm 0.44^{b,c}$	$8.36 \pm 0.57^{b,c}$	+ +	$10^{6.2}$	$10^{5.9}$
210 (IIa)	0.06 ± 0.01^c	$0.86 \pm 0.26^{b,c}$	32.7 ± 2.54^a	14.3 ± 1.91^a	+ +	$10^{5.4}$	$10^{5.5}$
40 (IIb)	0.09 ± 0.01^a	$0.77 \pm 0.22^{b,c}$	2.50 ± 0.48^c	2.76 ± 0.50^d	+	$10^{5.4}$	$10^{5.2}$
172 (IIb)	0.09 ± 0.01^a	0.50 ± 0.09^c	2.20 ± 0.39^c	2.50 ± 0.45^d	+	$10^{5.2}$	$10^{5.1}$
179 (IIb)	0.09 ± 0.01^a	0.61 ± 0.18^c	2.67 ± 0.70^c	2.53 ± 0.21^d	+	$10^{5.4}$	$10^{5.6}$
230 (IIb)	0.08 ± 0.01^a	0.47 ± 0.16^c	2.25 ± 0.78^c	1.76 ± 0.31^d	+	$10^{5.5}$	$10^{5.7}$
34 (III)	0.07 ± 0.01^b	1.73 ± 0.06^a	$3.52 \pm 0.92^{b,c}$	$5.18 \pm 1.66^{c,d}$	+ +	$10^{6.6}$	$10^{6.1}$
402 (IV)	$0.06 \pm 0.01^{b,c}$	$1.41 \pm 0.08^{a,b}$	6.42 ± 1.38^b	9.74 ± 2.22^b	+ +	$10^{5.1}$	$10^{4.9}$
130/91 (wt)	$0.07 \pm 0.01^{b,c}$	$1.18 \pm 0.19^{a,b,c}$	$4.71 \pm 0.97^{b,c}$	$8.12 \pm 0.36^{b,c}$	+ +	$10^{5.1}$	$10^{5.0}$

*ROI production was analysed by the superoxide anion assay as described in Methods. Results are expressed as means ± SEM from quadruplicate wells from one of three independent experiments.

†Survival in serum was calculated by dividing the viable bacterial population after serum treatment by the initial population before serum treatment. A value of >1 was regarded as serum resistant and a value of <1 as serum sensitive. Values are means ± SEM for three trials.

‡Adhesion is expressed as percentage of bacteria still adherent after washing without gentamicin treatment (means ± SEM, $n = 4$).

§Internalization is expressed as percentage of input bacteria surviving after gentamicin treatment for 2 h (means ± SEM, $n = 4$).

‖ +, Little growth; + +, significant growth.

¶Calculated by the method of Reed & Muench (1938). A value of less than 10^6 was scored as virulent. Sample size was 10 fish per group ($n = 3$). i.m., intramuscular injection; i.p., intraperitoneal injection.

these phenotypes; eight such mutants were identified, and further grouped into four classes (Table 1).

One of these mutants (2A), which produced significantly less siderophore than the others, was grouped in class I. The colonies of parent strain PPD130/91 and all the other mutants exhibited a distinct wide orange halo on CAS agar, indicative of siderophore production, while a smaller halo was observed for mutant 2A.

The five mutants belonging to classes IIa (210) and b (40, 172, 179 and 230) were sensitive to serum. Two of these (210 and 230) were also partially motile (Table 1). The class III mutant (34) was unable to swarm and showed completely defective motility when compared to the wild-type. It also produced less catalase. The class IV mutant (402) was deficient only in catalase production. Brisk effervescence was associated with the breakdown of H_2O_2 by the wild-type while catalase-deficient mutants (34 and 402) generated significantly less effervescence.
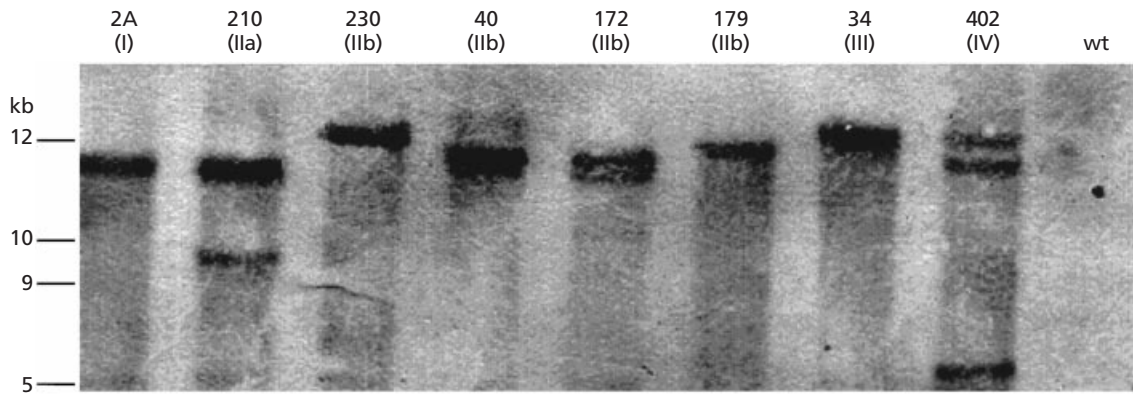
Fig. 1. Southern analysis of genomic DNA from *Ed. tarda* PPD 130/91 and the four classes of mutants. The *Eco*RV-digested DNA was separated on a 0·8% agarose gel and transferred to a nylon membrane. The blot was then hybridized with a biotinylated Tn*phoA* probe.

Further characterization of the Tn*phoA* mutants

All eight of the above mutants were Amp^s, suggesting that there was no suicide plasmid retention and integration.

The H$_2$O$_2$ inhibition zone tests showed that the catalase-deficient mutants 34 and 402 were more vulnerable to killing by H$_2$O$_2$ than the wild-type. The inhibition zones for mutants 34 and 402 were $10·3 \pm 0·3$ mm ($n = 3$) and $14·3 \pm 0·3$ mm ($n = 3$), respectively, when they were incubated overnight with disks containing 200 mM H$_2$O$_2$. Smaller inhibition zones were observed for the wild-type and other transposon mutants ($6·7 \pm 0·2$ mm, $n = 3$). All the mutants were analysed for their ability to induce production of reactive oxygen intermediates (ROI) in phagocytes. No significant differences were found between the catalase-deficient mutants and the wild-type (Table 2).

Mutants belonging to classes IIa and IIb were found to be serum-sensitive while the others were resistant to the gourami serum. Most of the mutants examined adhered to, and invaded, EPC cells at rates similar to that of the wild-type (Table 2). However, mutant 210 (class IIa) showed a significantly higher rate of adhesion (approximately seven times higher) and internalization (approximately two times higher) and hence differed from the mutants of class IIb, whose rate of internalization was significantly lower than that of the wild-type. Slow growth in phosphate-limiting medium was observed in all the class IIb mutants, compared to the wild-type.

Southern hybridization

The results of the Southern hybridization are shown in Fig. 1. All the mutants showed a single Tn*phoA* insertion, except for mutants 210 (class IIa) and 402 (class IV), which had two distinct transposon insertions each. The size of the third band identified for mutant 402 was smaller than that of the transposon (7·7 kb), and therefore should not be due to a third transposon insertion. The hybridized fragments of all the classes

were more than 8·0 kb in size. No band was found for the wild-type genomic DNA.

Sequence analysis

The insertion of Tn*phoA* in the mutants was characterized and the genes interrupted by the transposon were sequenced. Sequence analysis was done with predicted open reading frames found in the sequences obtained. As shown in Table 3, the corresponding sequences of mutant 2A (class I) were highly homologous to the arylsulfate sulfotransferase gene of *Enterobacter amnigenus* (E value 4e−46). The class IIb mutants had transposon insertions in genes homologous to the *pst* operon of *Enterobacter cloacae* and *E. coli*; two (40 and 172) of them were at different locations in the *pstS* gene (e−151 and e−100, respectively), one (230) in the *pstB* gene (e−126) and another (179) in the *pstA* gene (2e−20). The transposon insertion in mutant 34 (class III) was identified in a gene homologous to that of the paraquat-inducible catalase precursor gene of *Pseudomonas aeruginosa* (3e−58).

Attenuation of virulence in fish

Both intramuscular and intraperitoneal routes of delivery were used to determine the LD$_{50}$ values of the eight mutants. The dead fish had external signs of haemorrhage that were consistent with *Ed. tarda* infection. The LD$_{50}$ calculations indicated that mutants 2A (class I) and 34 (class III) were attenuated compared to the wild-type (Table 2). There was a 1·1 (intraperitoneal route) to 1·5 (intramuscular route) log increase in the LD$_{50}$ of mutant 34 when compared to the wild-type, while in the case of mutant 2A, the increase was about 1·0 log. The LD$_{50}$ values of mutants belonging to classes IIa and b were slightly higher than that of mutant 402 (class IV) and the wild-type.

Table 3. Sequence analysis of the potential virulence genes interrupted by Tn*phoA* in various transposon mutants of *Ed. tarda* PPD130/91

Mutant (and class)	Sequence accession no.	Predicted protein (aa)	Homologies to predicted encoded protein	E value (and % identity)*	Homologue accession no.
2A (I)	AF324343	221	Arylsulfate sulfotransferase (*astA*) of *Enterobacter amnigenus*	$4e-53$ (74%)	AF012826
40 (IIb)	AF324338	314	Phosphate-binding protein (*pstS*) of *Enterobacter cloacae* (Kusaka *et al.*, 1997)	$e-151$ (81%)	BAA22861
172 (IIb)	AF324342	211	Phosphate-binding protein (*pstS*) of *Enterobacter cloacae* (Kusaka *et al.*, 1997)	$e-100$ (83%)	BAA22861
179 (IIb)	AF324339	61	Phosphate-transport permease protein (*pstA*) of *Escherichia coli* (Amemura *et al.*, 1985)	$2e-20$ (77%)	P07654
230 (IIb)	AF324340	259	Phosphate-transport ATP-binding protein (*pstB*) of *E. coli* (Amemura *et al.*, 1985)	$e-126$ (87%)	P07655
34 (III)	AF324341	131	Catalase precursor [paraquat-inducible catalase isozyme B (*katB*)] of *Pseudomonas aeruginosa* (Brown *et al.*, 1995)	$3e-58$ (78%)	Q59635

* Sequence analysis was done with predicted open reading frames found in the sequences obtained. The E value indicates the probability of the match; a match with an E value of $1e-5$ and below was taken to be significant (i.e. the match is not due to chance). '% identity' indicates the number of identical amino acids over the span of amino acids.

DISCUSSION

Tn*phoA* library

Apart from constructing a library of PhoA$^+$ fusion mutants for our on-going screening of virulence proteins in *Ed. tarda*, this work has identified several genes associated with pathogenicity. The virulent parent strain, PPD130/91, is positive for several factors speculated as being involved in virulence factors of *Ed. tarda*, such as siderophores, motility and serum resistance (Janda *et al.*, 1991b). It also produces catalase, an enzyme with an important role in bacterial pathogenesis (Miller & Britigan, 1997). Only eight transposon mutants were isolated with deficiencies in the above four known phenotypes and no identical mutants were found among the 440 clones during screenings. Our results suggested that the size of our library was quite sufficient. We were able to isolate corresponding mutants as long as the screening systems were available. Furthermore, we did not isolate identical mutants after four rounds of screening, suggesting that our library is unique. This library will be screened further for other unknown virulence factors of *Ed. tarda*. For example, individual mutants will be screened in fish to identify any that are highly attenuated. This *in vivo* screening will be valuable for identifying important virulence genes in *Ed. tarda* pathogenesis.

Characterization of Tn*phoA* mutants

The eight mutants isolated from the library were grouped into four classes. Class I consisted of the attenuated and siderophore-deficient mutant 2A. Classes IIa and b consisted of the slightly attenuated and serum-sensitive mutants 40, 172, 179, 210 and 230. Two of these (210 and 230) were also partially defective in motility. Class IIb mutants (40, 172, 179 and 230) were pleiotropic, exhibiting lower internalization rates and poor growth in phosphate-limiting medium. They had transposon insertions in the *pst* operon, homologous to that of *Ent. cloacae* and *E. coli*. Mutant 210 (class IIa) differed from class IIb mutants, in having the Tn*phoA* insertion outside the *pst* operon. This mutant also showed a significantly higher rate of adhesion and internalization into EPC cells. The class III mutant was attenuated, non-motile and deficient in catalase production; it had the transposon insertion in a gene homologous to a catalase precursor gene (*katB*) of *P. aeruginosa*. The class IV mutant was deficient only in catalase production but it retained its virulence.

Siderophores and bacterial virulence

Mutant 2A (class I), deficient in siderophore production, exhibited lower virulence than the parent strain PPD130/91. The siderophore-mediated iron acquisition system is common to all species of *Enterobacteriaceae* (Dhaenens *et al.*, 1999) including *Ed. tarda* (Kokubo *et al.*, 1990), and is necessary for bacterial survival in the host. Since most pathogens produce siderophores to secure iron from the host (Weinberg, 1998), the lack of siderophore synthesis may reduce pathogenicity, as seen in mutant 2A and similar studies done on *Neisseria gonorrhoeae* (Yancey & Finkelstein, 1981) and *Vibrio anguillarum* (Wertheimer *et al.*, 1999). Iron is important for the process of bacterial colonization and survival within the host (Mietzner & Morse, 1994). However, iron metabolism may not be the only limiting factor for

establishing septicaemia, as most *Ed. tarda* isolates, including avirulent strains, produce siderophores (Thune *et al.*, 1993).

Mutant 2A showed transposon interruption in a gene with significant homology to the gene encoding the arylsulfate sulfotransferase protein, AstA, of *Ent. amnigenus* (Baek *et al.*, 1996). This enzyme catalyses the transfer of sulfate groups from phenylsulfate esters to phenolic compounds (Konishi-Imamura *et al.*, 1995) and may play an important role in the metabolism of phenolic compounds (Konishi-Imamura *et al.*, 1994). It is presently not clear how arylsulfate sulfotransferase affects the production of siderophores in *Ed. tarda*. Enterobactin is a catecholate (phenolate) siderophore produced by enteric bacteria (Neilands, 1981). Interruption of the arylsulfate sulfotransferase gene as in mutant 2A may directly or indirectly affect the biosynthesis of siderophores such as enterobactin in *Ed. tarda*. Since siderophores are essential for intracellular survival of *Mycobacterium tuberculosis* (DeVoss *et al.*, 2000), it could be speculated that mutant 2A may have a reduced virulence in fish because it is deficient in siderophore production.

Catalase and bacterial virulence

The production of ROI such as superoxide and H_2O_2 is crucial for the optimal microbicidal activities of phagocytes in host defence (Miller & Britigan, 1997). In response, bacteria have developed complex strategies including cell-surface structures such as lipopolysaccharide and peptidoglycan, and production of enzymes such as catalase, to overcome the hostile environments. Our catalase-deficient mutants (34 and 402) were more vulnerable to killing by H_2O_2 than the wild-type, suggesting that catalase might be one of the virulence factors in *Ed. tarda*. The ability of the eight mutants to induce ROI production by phagocytes was also determined. There was no direct correlation between catalase deficiency and increased induction of ROI production in the mutants. The Tn*phoA* library can be used further to screen for mutants sensitive to phagocyte-mediated killing. The resulting mutants and corresponding genes will be valuable for understanding the intimate relationship between phagocytes and intracellular pathogens.

Motility and bacterial virulence

Ed. tarda PPD130/91 exhibited pronounced motility when assayed on soft agar. Mutant 34 (class III) was deficient in motility and was also attenuated in fish. Its motility deficiency may have hindered its spread and colonization in the host. However, this mutant was not impaired in its ability to adhere to, and be internalized into, EPC cells. Other studies showed that non-motile mutants of *Aeromonas hydrophila* and *V. anguillarum* were unable to interact with fish cells (Merino *et al.*, 1997; Ormonde *et al.*, 2000) and exhibited reduced pathogenicity in fish (Norqvist & Wolf-Watz, 1993;

O'Toole *et al.*, 1996). On the other hand, a partially motile mutant 210 (class IIa) obtained in this study, was found to be hyperadhesive and hyperinvasive, suggesting that motility might influence bacterial interaction with fish cells.

The reduction in catalase production and virulence in the non-motile mutant (34) might be due to a mutation of the catalase precursor gene homologous to the *katB* gene of *P. aeruginosa* (Brown *et al.*, 1995). Since bacterial colonization of host tissue is affected by motility (Comolli *et al.*, 1999) and also catalase activity (Visick & Ruby, 1998), mutant 34 may be attenuated due to its deficiency of both motility and catalase production. However, it cannot be concluded which of the two phenotypes are important for bacterial virulence since the catalase-deficient, but motile and non-attenuated mutant (402, class IV), showed double insertions of Tn*phoA*. Further studies are needed to understand the pleiotropic nature and polar effects of the transposon mutations. Our results on bacterial motility and virulence need to be investigated further by green fluorescent protein (GFP) tagging using an immersion challenge model in blue gourami, to determine whether mutants defective in motility can invade and colonize fish.

Serum-sensitive mutants

Mutants 40, 172, 179 and 230 (class IIb), having Tn*phoA* insertion in the *pstS*, *pstA* and *pstB* genes of the *pst* operon, were identified as being deficient in several phenotypes: serum resistance, motility, internalization into EPC cells and reduced growth in phosphate-limiting medium. Serum resistance of *E. coli* depends on the *pst* operon (Daigle *et al.*, 1995) and perhaps also in *Ed. tarda* as seen in this study. The partial motility of mutant 230 may perhaps be due to its mutation in a gene homologous to the *pstB* gene of *Ent. cloacae*, which regulates phosphate transport, since both bacterial chemotaxis (Kusaka *et al.*, 1997) and motility (Rashid & Kornberg, 2000) require phosphorus. However, this defect in motility did not abrogate virulence in fish. Mutants in class IIb had a lower internalization rate, compared to the wild-type (Table 2). Although the precise mechanism of bacterial internalization remains unknown, it may be regulated by the *pst* operon whose mutation can also retard growth in phosphate-limiting medium.

Under phosphate-limiting conditions, phosphate assimilation is aided by the *pst* operon. Since mutation in this operon resulted in considerable changes in several phenotypes, perhaps this operon and phosphorus uptake may be important in the regulation of bacterial growth and metabolism (Wanner, 1987; Muda *et al.*, 1992). The regulation of *Ed. tarda* pathogenesis by the inorganic phosphate level is not known but this nutrient has been implicated in bacterial virulence (Demain, 1994), and virulence factors such as haemolysin with phospholipase C (PLC) activity are induced in low-phosphate environments (Gray *et al.*, 1982). Our future experiments will focus on virulence factors that are specifically expressed under phosphate-limiting conditions.

Conclusions

In conclusion, Tn*phoA* transposon mutagenesis was used to construct a library of fusion mutants from which several genes were identified that might play a role in *Ed. tarda* virulence. Attenuation of one or more of these virulence factors can be used to develop live vaccines to combat edwardsiellosis. More work needs to be done, especially in terms of characterizing and screening our remaining library of fusion mutants to identify more virulence genes. The information generated from this study will contribute to a better understanding of how *Ed. tarda* causes diseases in fish and other animals.

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