Isolation and characterization of adhesin-defective *TnphoA* mutants of septicaemic porcine *Escherichia coli* of serotype O115:K⁻:F165

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Non-enterotoxigenic porcine *Escherichia coli* strains belonging to the serogroup O115 have been associated with septicaemia and diarrhoea. Putative factors important in the pathogenicity of *E. coli* of serogroup O115 include fimbrial antigen F165, haemagglutination (MRHA), lipopolysaccharide, serum resistance, capsule and production of aerobactin. Using *TnphoA* transposon insertion mutagenesis, two classes of mutants were obtained from *E. coli* of serotype O115:F165 with respect to the phenotypic expression of fimbrial antigen F165 and MRHA of sheep erythrocytes: class I, F165⁻MRHA⁻, serum resistant; class II, F165⁺MRHA⁻, serum resistant. In a chicken lethality model, class I mutants were either virulent or of intermediate virulence, while class II mutants were of intermediate virulence. Alkaline phosphatase activity of class I and class II *TnphoA* mutants showed similar environmental regulation to that of fimbrial antigen F165. Moreover, class I and class II mutants were mutated in the *prs*-like locus, and lacked a 18.5 kDa and/or a 17.5 kDa fimbrial band.

Introduction

Certain pathogenic *Escherichia coli* strains cause localized or systemic extra-intestinal diseases such as urinary tract infection, septicaemia, meningitis and polyserositis (Ørskov & Ørskov, 1985). The virulence mechanisms of such strains appear to be complex and are only partially understood. These are thought to include the ability to colonize mucosal surfaces, to invade extra-intestinal tissues, to resist the bactericidal effect of complement, to survive and multiply in body fluids with low concentrations of available iron, to escape phagocytosis and intracellular killing by phagocytes, and to induce tissue damage and an inflammatory response by release of cytotoxins or endotoxins. Several virulence attributes, including lipopolysaccharide (e.g. O18) and capsule (e.g. K1) (Pluschke *et al.*, 1983), fimbriae and other surface antigens mediating mannose-resistant haemagglutination (MRHA) (e.g. P, AFA, S, Vir, 31A and F165) (Fairbrother *et al.*, 1986; Girardeau *et al.*, 1988; Hacker, 1989), cytotoxins (e.g. haemolysin, Vir cytotoxin, cytolethal necrotoxin) (Morris *et al.*, 1982; Korhonen *et al.*, 1985), aerobactin (Carbonetti *et al.*, 1986), ColV and

Vir plasmids (Morris *et al.*, 1982; Agüero & Cabello, 1983), have been associated with the ability of *E. coli* to cause extra-intestinal infections.

E. coli of serogroup O115 induce septicaemia and polyserositis in calves and piglets (Fairbrother *et al.*, 1986, 1988a, 1989). These strains express a fimbrial antigen complex, F165, consisting of fimbrial components F165₁ and F165₂, the former demonstrates binding specificity for the Forssman antigen found on sheep erythrocytes (Fairbrother *et al.*, 1986; J. M. Fairbrother and others, unpublished). They possess the *pap* and *sfa* operons, which are commonly associated with *E. coli* isolated from extra-intestinal diseases (Harel *et al.*, 1991a); they also possess the aerobactin system, are resistant to the bactericidal effect of serum, are non-haemolytic and do not produce verotoxin (Fairbrother *et al.*, 1988a). The presence of fimbrial antigen complex F165 and binding to the galactose-*N*-acetyl- α (1-3)galactose-*N*-acetyl moiety have been associated with production of septicaemia in newborn piglets experimentally inoculated with serogroup O115 strains (Fairbrother *et al.*, 1989). However, the role of this fimbrial antigen and its adhesin in the development of disease has not been ascertained.

Transposon mutagenesis has been used to identify virulence determinants in a variety of pathogens. Manoil & Beckwith (1985) have described a bacterial transposon

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Abbreviations: MRHA, mannose-resistant haemagglutination; XP, 5-bromo-4-chloro-3-indolyl phosphate.

Tn5IS50_L::*phoA* (Tn*phoA*), a modified form of Tn5, which carries the *E. coli* alkaline phosphatase structural gene without the natural promoter or signal sequence. After random insertion of Tn*phoA* into genomic DNA, those insertions that yield in-frame fusions between a target gene and *phoA* encode hybrid proteins that have a carboxy-terminal fragment of PhoA fused to an amino-terminal portion of the target protein product (Manoil & Beckwith, 1985). These hybrid proteins display alkaline phosphatase activity only if the target gene encodes an extracytoplasmic protein and so provides the requisite signals for transport of the carboxy-terminal *phoA* gene product into the periplasmic space (Manoil & Beckwith, 1985). Because most bacterial virulence determinants are expressed at the cell surface, this technique selects for Tn*phoA* insertions into such genes (Taylor *et al.*, 1987, 1989; Finlay *et al.*, 1988; Miller *et al.*, 1989). Thus, using the Tn*phoA* system, isogenic strains of a pathogenic F165-positive strain of *E. coli* of serogroup O115 were produced, with respect to two putative virulence determinants, mannose-resistant haemagglutination of sheep erythrocytes and production of the F165 fimbrial antigen, in order to evaluate the role of these determinants in the pathogenesis of *E. coli* septicaemia.

Methods

Bacterial strains and plasmids. *Escherichia coli* strain 5131 is a wild-type strain (O115:K⁻:H51:F165) isolated from the intestinal contents of a diarrhoeic piglet. Strain 5131 haemagglutinates sheep erythrocytes but not bovine erythrocytes, produces aerobactin, produces Colicin V, and is serum resistant. Strain 5131 was found to have sequences related to the *pap* and *sfa* operons (Harel *et al.*, 1991a). *E. coli* strain SM10 possessing plasmid pRT733 carrying Tn*phoA* was used for transposon mutagenesis (Taylor *et al.*, 1989). Plasmids pCJ7 (Harel *et al.*, 1992) and pANN801-13 (Hacker *et al.*, 1985) were used as the source of DNA probes for fimbrial genes coding for F165₁, a Prs-like fimbriae, and for S fimbriae, respectively. Bacteria were cultivated in L-broth or on L-agar plates containing 1.5% (w/v) agar (Miller, 1972). F165-positive strains were grown on minimal medium (MD-1) for the maximal expression of F165 fimbrial antigen (Fairbrother *et al.*, 1988b). Selective pressure was imposed by adding 50 µg kanamycin ml⁻¹ to the medium.

Mutagenesis. Mutations were induced by random insertion of the Tn*phoA* sequence into chromosomal or plasmid DNA of *E. coli* strain 5131. The mutagenesis was accomplished by using the plasmid pRT733 which carries the Tn*phoA* insertion in donor *E. coli* strain SM10 (λ pir) (Taylor *et al.*, 1989; Miller & Mekalanos, 1988). pRT733 is a suicide vector since it cannot replicate in the absence of the protein encoded by the λ pir transducing phage, but it can be mobilized from strain SM10 (λ pir) into strain 5131 during mating. Fresh colonies of donor strain SM10 carrying the vector pRT733 were mated with recipient strain 5131 (Sm^rAp^r) in LB plates and then incubated at 37 °C for 16 h. After dilution, the mating mix was then spread onto agar containing streptomycin and kanamycin. Therefore, selection for Km^r and Sm^r resulted in the transposition of Tn*phoA* into the chromosome or plasmid of the Sm^r recipient. The indicator XP (5-bromo-4-chloro-3-indolyl phosphate) was included in the agar to detect those transposi-

tions that resulted in active *phoA* gene fusions in the recipient. Of approximately 2000 Km^r Sm^r transposon insertions, 1% were found to produce blue colonies on agar in the presence of alkaline phosphatase substrate XP.

DNA manipulation techniques. Chromosomal DNA was isolated according to the method of Marmur (1961). The method of Birnboim & Doly (1979) was followed for the isolation of plasmid DNA. Plasmid DNA was extracted and purified by ultracentrifugation in caesium chloride/ethidium bromide gradients (Maniatis *et al.*, 1982). DNA was digested with the appropriate restriction endonucleases under conditions specified by the manufacturer (Pharmacia-LKB). Electrophoretic analysis of DNA was done using 0.7% agarose gel and Tris/borate buffer (0.045 M-Tris/borate, 0.001 M-EDTA) (Maniatis *et al.*, 1982). Restriction fragments of plasmid DNA were purified by extraction from low-gelling-temperature agarose after electrophoresis (Harel *et al.*, 1991b).

Southern hybridization, gene probes and radioactive labelling. The transfer of DNA fragments from agarose gels to nitrocellulose filters, washing and autoradiography were done as described by Southern (1975). Stringent conditions were used for the washing procedure. The filters were washed in 2 × SSC (1 × SSC is 0.15 M-NaCl + 0.015 M-sodium citrate) twice for 5 min at room temperature each time, then in 2 × SSC with 0.5% SDS twice for 30 min each time at 65 °C, and finally in 0.1 × SSC twice for 5 min each time at room temperature. Fimbrial probes representing the full F165₁ operon (*Bam*HI fragment from pCJ7, encoding the F165₁ operon) (Harel *et al.*, 1992) and *sfa* operon (*Eco*RV fragment from pANN801-13) (Hacker *et al.*, 1985) were used in this study. Appropriate DNA fragments were cut from the gel, purified, and radiolabelled with [α-³²P]dCTP using an oligonucleotide random priming labelling kit (Pharmacia-LKB) according to the manufacturer's instructions (Harel *et al.*, 1991b).

Enzyme activity. To measure the amount of alkaline phosphatase produced by the gene-fusion-carrying strains, bacteria were grown in medium for 16 h. Cells were harvested and suspended in 1 M-Tris/HCl (pH 8.0), a sample was diluted 1/20 and permeabilized with SDS and chloroform. The alkaline phosphatase activity was expressed in enzyme units per OD₆₆₀ unit (Miller, 1972; Taylor *et al.*, 1989).

Detection of F165 fimbrial antigen. Production of F165 antigen by bacteria grown on MD-1 agar was determined using an immunodot technique (Contrepolis *et al.*, 1989).

Isolation of fimbriae, SDS-PAGE and immunoblotting. Crude fimbrial extracts were prepared from bacteria grown on MD-1 plates as described previously (Fairbrother *et al.*, 1988b). After treatment of the fimbrial samples by boiling for 5 min in 10 mM-Tris/HCl (pH 7.8) containing 4% (w/v) SDS, 0.01 ml 2-mercaptoethanol, 0.2 ml glycerol and 0.002% bromophenol blue, the samples were run on slab gels as described previously (Fairbrother *et al.*, 1988b). Western blotting (immunoblotting) was done by the method of Towbin *et al.* (1979). Crude fimbrial extracts from wild-type *E. coli* strain 5131 and from Tn*phoA* mutants were electrophoresed in the presence of SDS (0.1%, w/v) on 15% (w/v) polyacrylamide gels. Following electrotransfer to nitrocellulose, the filters were reacted with anti-F165 and anti-PhoA sera to identify the fimbrial bands and *phoA* gene fusion products, respectively.

Gels were electroblotted for 16 h at 30 V on nitrocellulose using cold electroblotting buffer in 20% (v/v) methanol containing 25 mM-Tris and 192 mM-glycine. The filters were saturated with 5% (w/v) skim milk and incubated for 90 min at 25 °C with anti-F165 or anti-PhoA serum in Tris-buffered saline (TBS; 20 mM-Tris/HCl, pH 7.5, 500 mM-NaCl) containing 0.05% (v/v) Tween 20 (TTBS), plus 2.5% (w/v) skim milk. Filters were then incubated for 1 h at 25 °C in a 1/5000 dilution of biotinylated goat anti-rabbit immunoglobulin G. After a short wash in

TTBS, the filters were incubated in a 1/3000 dilution of peroxidase-labelled avidine. Finally, filters were washed twice in TTBS and twice in TBS and reacted with hydrogen peroxide substrate and 4-chloronaphthol chromogen.

Polyclonal antibodies were generated against FPLC-purified F165 antigen complex from the F165-antigen-producing *E. coli* strain 4787 and against alkaline phosphatase by injection of 10 µg aliquots of purified proteins into rabbits as described previously (Fairbrother *et al.*, 1986). To enhance the specificity of immunodetection of hybrid proteins, the anti-F165 and anti-PhoA antibodies were absorbed against whole cells of strain 4787 grown at 18 °C or of strain CC118 (*ΔphoA*), respectively (Manoil & Beckwith, 1985; Coulton *et al.*, 1988).

Immunoelectron microscopy. Drops of a washed bacterial suspension were placed on Formvar-coated grids for 3 min. The grids were then placed sequentially on drops of phosphate-buffered saline, pH 7.4 (PBS; 0.2 M-NaH₂PO₄, 0.2 M-Na₂HPO₄, 0.1 M-NaCl) with 1% (w/v) ovalbumin for 5 min and a suitable dilution of rabbit anti-F165 serum for 30 min. Grids were washed and placed on drops of colloidal gold particles (5 nm) conjugated to protein A (Sigma) for 30 min. After a final washing step, the grids were stained with 1% (w/v) phosphotungstic acid (pH 7.0) and examined with an electron microscope (Philips 201) at an accelerating voltage of 60 kV (Broes *et al.*, 1988).

Detection of F165 adhesin. The presence of F165 adhesin was determined by a haemagglutination assay using sheep erythrocytes with 2% (w/v) mannose as described previously (Fairbrother *et al.*, 1986).

Hydrophobicity. The hydrophobicity of bacterial cells was determined by the method of Lindahl *et al.* (1981). Hydrophobicity values are reported as the molar concentrations of (NH₄)₂SO₄ required to cause autoagglutination of the bacterial suspensions.

Serum bactericidal assay. Bacterial survival in serum was examined using a bactericidal assay adapted from Taylor & Kroll (1983). Briefly, a 10-fold dilution of overnight culture was resuspended in TBS and incubated under agitation for 1.5 h. A 10 µl volume of this culture was resuspended in 990 µl gelatin/Veronal-buffered saline plus magnesium and calcium ions (pH 7.35) (GVB²⁺) (Taylor & Kroll, 1983), to obtain

approximately 10⁷ c.f.u. ml⁻¹. A 100 µl volume of this suspension was added to 900 µl normal rabbit serum. After mixing, the bacterial suspensions were incubated at 37 °C for 3 h. Viable counts were estimated at hourly intervals by plating 100 µl of the mixture on brain heart infusion agar plates (Difco). Strains demonstrating a viable count of >100% of the inoculum after 3 h were considered to be resistant to serum; strains demonstrating a viable count of <100% but >1% of the inoculum were considered to have intermediate sensitivity to serum; and strains demonstrating a viable count of <1% of the inoculum were considered to be sensitive to serum.

Lethality test. An overnight tryptic soy broth (Difco) culture was centrifuged and resuspended in PBS. A 0.5 ml volume of this suspension was inoculated subcutaneously into 1 d-old chickens obtained from a local hatchery. The 50% lethal dose (LD₅₀) of each strain was determined by inoculating groups of five chickens with doses of bacteria between 10⁸ to 10⁶ c.f.u. ml⁻¹ in 2-fold dilution steps. Bacterial counts were estimated using a spread plate method on brain heart infusion agar (Difco). Chickens were observed for 2 d, mortalities were recorded and the 50% lethal dose (LD₅₀) was calculated (Reed & Muench, 1938).

Results

Isolation and characterization of *TnphoA* gene fusions

After random insertion of *TnphoA* into the genome of F165-antigen-positive *E. coli* strain 5131, colonies were screened for in-frame *TnphoA* insertions in genes encoding exported proteins on LB agar containing XP. Of 2000 Km^r Sm^r transposon insertion mutants, 1% were found to produce blue colonies in the presence of alkaline phosphatase XP substrate. Mutants were screened for loss of the haemagglutination phenotype using sheep

Table 1. Characteristics and virulence in chickens of *TnphoA* mutants of *E. coli* strain 5131

The results for MRHA, presence of F165 antigen, hydrophobicity, serum sensitivity and LD₅₀ are representative of averages obtained from triplicate assays.

	Mutant no.	MRHA*	F165 antigen†	Hydrophobicity value (M)‡	Survival in serum (%)§	log ₁₀ LD ₅₀ ¶	Virulence
Wild-type	5131	+	+	0.125	227	6.8	Virulent
Mutants	M9	—	—	4	117	6.8	Virulent
	M43	—	—	2	125	6.8	Virulent
	M48	—	—	2	138	6.7	Virulent
	15	—	+/-	4	10	7.2	Intermediate
	6	—	+	ND	4.7	7.3	Intermediate
	9	—	+	0.25	29	7.9	Intermediate
	17	—	+	0.5	5.8	7.8	Intermediate
	18	+	+	ND	1.2	7.2	Intermediate
	2	+	+	0.5	0.8	8.9	Avirulent

* MRHA⁻, no haemagglutination activity; MRHA⁺, haemagglutination activity.

† As tested by immunodot. +, strongly positive; +/-, weakly positive; —, negative.

‡ Molar concentration of (NH₄)₂SO₄ required to cause autoagglutination of bacterial suspensions. ND, Not determined.

§ Bacteria (10⁶ c.f.u. ml⁻¹) in the late exponential growth phase were incubated in the presence of 90% (v/v) fresh rabbit serum, after growth in MD-1 medium. Results are expressed as the percentage of initial inoculum of each strain after 3 h incubation.

¶ Chickens (1-d-old) were injected subcutaneously with different dilutions of bacteria.

Fig. 1

Fig. 1. Southern blot analysis of genomic DNA from 10 mutant strains digested with *Bam*HI/*Eco*RV. The probe was a 1.9 kb *Hind*III-*Bam*HI *TnphoA*-derived fragment that in *Bam*HI/*Eco*RV double digests hybridizes to the upstream fragment (relative to transcription of the fusion) more strongly than to the downstream fragment. Lane 1, pRT733 (carrying *TnphoA*); lane 2, wild-type strain 5131; lane 3, mutant 2 (serum sensitive); lane 4, mutant 6 (MRHA⁻F165⁺); lane 5, mutant 15 (MRHA⁻F165^{+/+}); lane 6, mutant 9 (MRHA⁻F165⁺); lane 7, mutant 18 (intermediate serum resistance); lane 8, mutant 19 (MRHA⁻F165⁺); lane 9, mutant 29 (MRHA⁻F165⁺); lane 10, mutant M9 (MRHA⁻F165⁻); lane 11, mutant M43 (MRHA⁻F165⁻); lane 12, mutant M48 (MRHA⁻F165⁻).

Fig. 2

Fig. 2. Southern blot analysis of genomic DNA from 10 mutant strains digested with *Bam*HI/*Eco*RV. The probe was a 9 kb *Bam*HI fragment derived from plasmid pCJ7, a *prs*-like operon, coding for F165_i. Lane 1, wild-type strain 5131; lane 2, mutant 2 (serum sensitive); lane 3, mutant 6 (MRHA⁻F165⁺); lane 4, mutant 15 (MRHA⁻F165⁻); lane 5, mutant 9 (MRHA⁻F165⁺); lane 6, mutant 18; lane 7, mutant 19 (MRHA⁻F165⁺); lane 8, mutant 29 (MRHA⁻F165⁺); lane 9, mutant M9 (MRHA⁻F165⁻); lane 10, mutant M43 (MRHA⁻F165⁻); lane 11, mutant M48 (MRHA⁻F165⁻).

erythrocytes, F165 antigen production using immunodot assay with anti-F165 serum, and serum sensitivity using the serum bactericidal assay. At least two distinct classes of alkaline-phosphatase-secreting mutants were observed with respect to haemagglutination and F165 antigen production (Table 1): class I (mutants M9, M43, M48, 15), F165⁻MRHA⁻, serum resistant (except mutant 15); class II (mutants 6, 9, 17, 19, 29), F165⁺MRHA⁻, intermediate serum sensitivity. Mutants 19 and 29 were class II mutants not shown in Table 1, but had properties similar to that of mutant 6. Mutant 18 was F165⁺MRHA⁺, and of intermediate serum sensitivity. Mutant 2 was F165⁺MRHA⁺, serum sensitive and avirulent.

Mapping of *TnphoA* insertions

As restriction endonuclease *Eco*RV does not cut within transposon *TnphoA*, Southern hybridization of *Eco*RV-digested chromosomal DNA probed with an internal *Hind*III fragment of *TnphoA* was performed for each of the mutant strains (data not shown). This analysis verified that only single *TnphoA* insertions were present in most of the tested mutant strains. Moreover, single *TnphoA* insertions were present in distinct *Eco*RV fragments ranging from 9 to 20 kb [including the inserted

TnphoA (7.6 kb)]. One MRHA⁻F165⁻ mutant, mutant 15, possessed two *Eco*RV fragments which hybridized with the *TnphoA* DNA probe indicating the presence of a cointegrate or a double insertion of *TnphoA*.

Mutants were characterized with respect to the chromosomal DNA digestion patterns obtained with *Bam*HI, which cuts once in *TnphoA* (data not shown), and with *Bam*HI and *Eco*RV (Fig. 1). The DNA probe was a 1.9 kb *Hind*III-*Bam*HI *TnphoA*-derived fragment that with *Bam*HI/*Eco*RV double digests will hybridize to the upstream fragment (relative to transcription of the gene fusion) more strongly than to the downstream fragment (Taylor *et al.*, 1988). After chromosomal DNA double digests with *Bam*HI/*Eco*RV, the class I MRHA⁻F165⁻ mutants M9, M43, and M48 (Fig. 1, lanes 10, 11, and 12, respectively) presented a distinct hybridization pattern with an upstream band of 5–4 kb and a downstream band of 4–7 kb. The class I MRHA⁻F165⁻ mutant, mutant 15 (Fig. 1, lane 5), revealed four hybridizing bands, due to a double *TnphoA* insertion or to a cointegrate structure. Class II MRHA⁻F165⁺ mutants demonstrated a hybridization pattern with an upstream band of 5–5.8 kb and a downstream band of 4–4.5 kb (Fig. 1, lanes 4, 6, 8, 9). The serum-sensitive mutant 2 had a 6 kb upstream band and a 2.8 kb downstream band (Fig. 1, lane 3).

Table 2. Regulation of expression of *TnphoA* gene fusions in *E. coli* strain 5131 by environmental modulation

Alkaline phosphatase (PhoA) activity was measured in permeabilized cells and is expressed in enzyme units per OD₆₆₀ unit. The data are representative averages of duplicate assays.

Strain or mutant*	Alkaline phosphatase activity					
	MD-1 37 °C/18 °C	Ratio of PhoA activity at 37 °C/18 °C	MD-1 + 5 mM- alanine	Ratio of PhoA activity of cells grown on MD-1/MD-1 + alanine	MD-1 + 10 g glucose l ⁻¹	Ratio of PhoA activity of cells grown on MD-1/MD-1 + glucose
5131	0.18/0.54	0.3	0.4	0.45	0.18	1.0
Class I { M9 (MRHA ⁻ F165 ⁻)	40.31/4.82	8.36	17.2	2.3	29.1	1.4
M43 (MRHA ⁻ F165 ⁻)	28.09/9.11	3.95	10.5	2.7	12.9	2.2
M48 (MRHA ⁻ F165 ⁻)	25.82/4.39	5.88	14.1	1.8	10.1	2.6
15 (MRHA ⁻ F165 ^{+/})	20.9/0.7	29.9	11.1	1.9	6.7	3.1
Class II { 6 (MRHA ⁻ F165 ⁺)	10.6/1.0	5.3	5.5	1.9	3.16	3.4
9 (MRHA ⁻ F165 ⁺)	10.9/1.3	8.4	5.9	1.8	6.2	1.75
17 (MRHA ⁻ F165 ⁺)	10.1/2.4	4.2	4.8	2.1	4.1	2.4

* MRHA⁻F165⁻, no haemagglutination activity, negative as tested by immunodot with anti-F165 serum; MRHA⁻F165⁺, no haemagglutination activity, positive as tested by immunodot with anti-F165 serum.

Although most mutants had a plasmid profile similar to that of wild-type strain 5131, mutant 18 showed a different plasmid profile. Moreover, hybridization with the *TnphoA* probe of a gel containing plasmid preparations of each of the mutants revealed that mutant 18 contained a high molecular mass plasmid which reacted with the probe (data not shown). Thus, only mutant 18 (MRHA⁺F165⁺, intermediate serum sensitivity) had a *TnphoA* mutation within one of the large plasmids harboured by strain 5131.

E. coli strain 5131 was shown previously to be genotypically positive for P and S probes (Harel *et al.*, 1991a). Southern blotting of *EcoRV*/*Bam*HI-digested chromosomal DNA from *E. coli* strain 5131 and the class I and class II mutants was done using probes representing the full *F165*₁ operon (*Bam*HI fragment from pCJ7, encoding the *F165*₁ operon) (Harel *et al.*, 1992) and the *sfa* operon (*EcoRV* fragment from pANN801-13) (Hacker *et al.*, 1985). The *F165*₁ operon shows a *Bam*HI/*EcoRV* restriction map similar to the *prs* and *pap-2* clones (Lund *et al.*, 1988; Karr *et al.*, 1989; Harel *et al.*, 1992). Therefore, the disappearance of specific bands could be mapped in the *F165*₁ mutants. The blots demonstrated that *TnphoA* insertions in the class I and class II mutants were in the *F165*₁ operon, since the distribution of some bands hybridizing to the *F165*₁ probe had changed with respect to those of strain 5131 (Fig. 2). Moreover, the disappearance of *F165*₁-specific bands and the pattern of bands corresponding to the *F165*₁ sequences adjacent to *TnphoA* indicated that *TnphoA* insertions in class I strains were within the 5' end region (genes *AHC*) of the *F165*₁ operon but were different insertions (Fig. 2, lanes 4, 9, 10, 11). *TnphoA* insertions in class II mutants are within the 3' end region

hybridizing with the *F165*₁ operon (Fig. 2, lanes 3, 5, 7, 8). For most class I MRHA⁻F165⁻ mutants, *TnphoA* insertions were clustered within a region of 3 kb, whereas class II MRHA⁻ mutants contained *TnphoA* insertions clustered within a region of 1.0 kb (Fig. 1). None of the *TnphoA* insertions were in the *sfa*-related sequences, since the mutants showed hybridization patterns with the *sfa* operon probe from pANN801-3 similar to that of the strain 5131 (data not shown).

Analysis of *PhoA* fusion products

The *PhoA* fusion products of the mutants were characterized by Western blotting using rabbit anti-alkaline phosphatase serum. The *PhoA* fusion products were similar in size or larger than alkaline phosphatase (data not shown). The sizes of the fusion products ranged from 50 to 85 kDa. The class I (MRHA⁻F165⁻) *PhoA* fusion products ranged from 50 to 85 kDa; class II (MRHA⁻) *PhoA* fusion products ranged from 55 to 65 kDa.

Alkaline phosphatase activity

The alkaline phosphatase (*PhoA*) activities of class I and II mutants grown under different conditions were assayed in permeabilized cells to study the regulation of expression of products of genes in which *TnphoA* had been inserted (Table 2). Class II mutants demonstrated similar alkaline phosphatase activity when grown on MD-1 medium at 37 °C, whereas class I mutants demonstrated greater *PhoA* activity than class II mutants. In all class I and II mutants, growth of cultures at 18 °C resulted in a decrease of *PhoA* activity of 4- to

Fig. 3. Western blot of fimbrial preparations from *TnpHoA* insertion mutants. The fimbrial preparations were separated by SDS-PAGE, transferred to nitrocellulose and reacted with rabbit anti-F165 serum. The arrows indicate the 18.5 and 17.5 kDa fimbrial bands. Lane 1, mutant 6 (MRHA⁻F165⁺); lane 2, mutant 9 (MRHA⁻F165⁺); lane 3, mutant M9 (MRHA⁻F165⁻); lane 4, wild-type 5131.

24-fold. Similarly, addition of 5 mM-alanine or 10 g glucose l⁻¹ to the growth medium resulted in a 2- to 3-fold repression of PhoA activity. Thus, PhoA fusions in class I and class II mutants are modulated by conditions similar to those that affect F165 antigen production.

F165 antigen expression in class I and class II mutants

Parent strain 5131 grown in MD-1 medium under optimal conditions for F165 fimbrial expression was hydrophobic (hydrophobicity value of 0.125 M) (Table 1), demonstrated two fimbrial bands of 18.5 kDa and 17.5 kDa on Western blots using anti-F165 serum and expressed fimbrial structures which were identified as F165 by immunogold-labelling experiments using anti-F165 serum (see Fig. 4*a*). In contrast, class I (MRHA⁻F165⁻) mutants were relatively non-hydrophobic (the

Fig. 4. Electron micrographs after colloidal gold immunolabelling using rabbit anti-F165 serum of (a) *E. coli* strain 5131, (b) mutant 6 (MRHA⁻F165⁺) and (c) mutant M9 (MRHA⁻F165⁻). Bars, 0.5 µm.

hydrophobicity values ranged from 2–4 M (Table 1), lacked the 17.5 kDa band and produced little of the 18.5 kDa band (Fig. 3). Moreover, class I mutants expressed very few fimbrial structures on the bacterial surface (Fig. 4c). Class II (MRHA⁻F165⁺) mutants lacked the 17.5 kDa band corresponding to one of the components of F165 fimbrial antigen but still produced the 18.5 kDa band (Fig. 3). These mutants were fimbriate on electron microscopy, in some cases less extensively than strain 5131 (Fig. 4b), reacted with F165 antiserum using the immunodot technique and demonstrated hydrophobicity values ranging from 0.25–1 M (Table 1). Serum-sensitive mutant 2, did not differ from the parent strain with respect to fimbrial characteristics (Table 1).

Virulence of the TnphoA mutants

Parent strain 5131 was serum-resistant and virulent in 1 d-old chickens, showing a log₁₀ LD₅₀ of 6.8 (Table 1). Similarly, most class I MRHA⁻F165⁻ mutants were serum resistant and virulent in chickens. In contrast, class II MRHA⁻F165⁺ mutants showed an intermediate sensitivity to serum and were moderately virulent in day-old chickens. Serum-sensitive mutant 2 was avirulent in day-old chickens.

Discussion

Serogroup O115 *E. coli* strains have been associated with porcine septicaemia and diarrhoea (Fairbrother *et al.*, 1988, 1989). Putative factors important in the pathogenicity of *E. coli* of serotype O115 include fimbrial antigen F165, haemagglutination, lipopolysaccharide, serum resistance, capsule and production of aerobactin (Fairbrother *et al.*, 1986, 1989). *TnphoA* insertion mutations which define genes involved in or associated with F165 fimbrial production were obtained as *phoA* fusions, thus indicating that the target gene encodes a secreted protein.

Like other fimbriae associated with extra-intestinal disease, F165 fimbriae are associated with haemagglutination patterns (Fairbrother *et al.*, 1986; Hacker, 1989), thus reflecting their ability to bind to receptors on the surface of host cells. Strain 5131 expressing the F165 fimbrial antigen recognizes the Forssman antigen of sheep erythrocytes (J. M. Fairbrother and others, unpublished). This property is associated with one of at least two fimbrial components of the F165 antigen complex, designated F165₁, which is encoded by a *prs*-like operon (Harel *et al.*, 1992). The property of adhesion to red blood cells was lost in class I and class II mutants (MRHA⁻F165⁺ and MRHA⁻F165⁻). By analogy to

other well characterized fimbrial systems (Hacker, 1989), the adhesion defect of these *E. coli* mutants seems related to F165₁ fimbrial adhesin. Strain 5131 expresses at least two fimbrial determinants with structural subunits of 18.5 kDa and 17.5 kDa. The mutants of both classes I and II have lost the 17.5 kDa band and the property of MRHA of sheep erythrocytes, which implies that this band is the structural subunit of fimbrial component F165₁ produced by strain 5131. The *TnphoA* insertions of mutants of class I and class II were located in the F165₁ operon, a *prs*-related operon (Lund *et al.*, 1988; Harel *et al.*, 1991a). These *phoA* fusions of class I and class II mutants (MRHA⁻F165⁺ and MRHA⁻F165⁻) showed similar environmental regulation to that of F165 as tested in a wild-type strain (Fairbrother *et al.*, 1988). Complementation and other genetic and biochemical analysis will be necessary to analyse the relationship between the *TnphoA* insertions and the absence of some components.

It is interesting to note that all class II mutants and mutant 15, a class I mutant, demonstrated intermediate sensitivity to serum, which correlated with a decrease in virulence in day-old chickens as compared to the parent strain. In contrast, most class I (MRHA⁻F165⁻) mutants remained serum resistant, although they did not multiply to as great an extent as the parent strain. The relationship, if any, between serum sensitivity and loss of the haemagglutination phenotype has to be elucidated. It was found that uropathogenic mutant strains that had lost the ability to produce haemolysin and adhesin demonstrated a marked decrease in resistance to normal human serum (Hacker *et al.*, 1988). It was suggested that different virulence factors were organized in functional 'virulence blocks'. Alternatively, in the case of class II mutants, structural modification of the adhesin or the fimbriae might promote binding of C3 or decrease protection of bacteria from complement-mediated membrane-attack complex.

Initially, the chicken model was used to rapidly screen a large number of mutants for loss of virulence. However, in such a model in which bacteria are introduced directly into the bloodstream, cell-surface structures enabling the bacteria to withstand the effects of complement present in the plasma are more important for bacterial multiplication and development of septicaemia. Bacterial colonization of a mucosal surface or other surface due to fimbrial adhesins would not play an important role in this model. Inoculation of young pigs by a more natural route, the gastro-intestinal tract, will enable us to evaluate more fully the role of the F165 adhesin in the establishment of infection and development of septicaemia.

Strains of enteric bacteria capable of causing bacteraemia and other extra-intestinal infections are often

significantly resistant to the bactericidal effect of serum (Taylor, 1988). Bacterial serum-resistance is a multifactorial characteristic and involves membrane surface structures which include outer-membrane proteins, lipopolysaccharides and capsular polysaccharides that protect bacteria from the complement-mediated membrane-attack complex. In the present study, *TnphoA* mutants were found that are highly susceptible to the bactericidal effect of serum. One serum sensitive mutant was avirulent in day-old chickens. Thus, such mutations may give insight into new approaches towards development of attenuated *E. coli* strains useful for vaccine development (Miller *et al.*, 1989).

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References

- AGÜERO, M. E. & CABELLO, F. C. (1983). Relative contribution of ColV plasmid and K1 antigen to the pathogenicity of *Escherichia coli*. *Infection and Immunity* **40**, 359–368.
- BIRNBOIM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids Research* **7**, 1513–1523.
- BROES, A., FAIRBROTHER, J. M., JACQUES, M. & LARIVIÈRE, S. (1988). Isolation and characterization of a new fimbrial antigen (CS1541) from a porcine enterotoxigenic *Escherichia coli* O8:KX105 strain. *FEMS Microbiology Letters* **55**, 341–348.
- CARBONETTI, N. H., BOONCHAI, S., PARRY, S. H., VAISANEN-RHEN, V., KORHONEN, I. K. & WILLIAMS, P. H. (1986). Aerobactin-mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. *Infection and Immunity* **51**, 966–968.
- CONTREPOIS, M., FAIRBROTHER, J. M., KAURA, Y. K. & GIRARDEAU, J.-P. (1989). Prevalence of CS31A and F165 surface antigens in *Escherichia coli* isolates from animals in France, Canada, and India. *FEMS Microbiology Letters* **59**, 319–324.
- COULTON, J. W., REID, G. K. & CAMPANA, A. (1988). Export hybrid proteins FhuA'–LacZ and FhuA'–PhoA to the cell envelope of *Escherichia coli* K-12. *Journal of Bacteriology* **170**, 2267–2275.
- FAIRBROTHER, J. M., LARIVIÈRE, S., LALLIER, R. (1986). New fimbrial antigen F165 from *Escherichia coli* serogroup O115 strains isolated from piglets with diarrhea. *Infection and Immunity* **51**, 10–15.
- FAIRBROTHER, J. M., LARIVIÈRE, S. & JOHNSON, W. M. (1988a). Prevalence of fimbrial antigens and enterotoxins in nonclassical serogroups of *Escherichia coli* isolated from newborn pigs with diarrhea. *American Journal of Veterinary Research* **49**, 1325–1328.
- FAIRBROTHER, J. M., LALLIER, R., LEBLANC, L., JACQUES, M. & LARIVIÈRE, S. (1988b). Production and purification of *Escherichia coli* fimbrial antigen F165. *FEMS Microbiology Letters* **56**, 247–252.
- FAIRBROTHER, J. M., BROES, A., JACQUES, M. & LARIVIÈRE, S. (1989). Pathogenicity of *Escherichia coli* O115:KV165 isolated from pigs with diarrhea. *American Journal of Veterinary Research* **50**, 1029–1036.
- FINLAY, B. B., STARNBACH, M. N., FRANCIS, C. L., STOCKER, B. A., CHATFIELD, S., DOUGAN, G. & FALKOW, S. (1988). Identification and characterization of *TnphoA* mutants of *Salmonella* which are unable to pass through a polarized MDCK epithelial cell monolayer. *Molecular Microbiology* **2**, 757–766.
- GIRARDEAU, J. P., DER VARTANIAN, M., OLLIER, J. L. & CONTREPOIS, M. (1988). CS31A, a new K88-related fimbrial antigen on bovine enterotoxigenic and septicemic *Escherichia coli*. *Infection and Immunity* **56**, 2180–2188.
- HACKER, J. (1989). Genetic determinants coding for fimbriae and adhesins of extra-intestinal *Escherichia coli*. *Current Topics in Microbiology and Immunology*, **151**, 1–28.
- HACKER, J., SCHMIDT, G., HUGHES, C., KNAPP, S., MARGET, M. & GOEBEL, W. (1985). Cloning and characterization of genes involved in production of mannose-resistant, neuraminidase-susceptible (X) fimbriae from uropathogenic O6:K15:H31 *Escherichia coli*. *Infection and Immunity* **47**, 434–440.
- HACKER, J., GOEBEL, W., HOF, H., KONIG, W., KONIG, B., SCHEFFER, J., HUGHES, C. & MARRE, R. (1988). Adhesins, serum resistance and cytotoxins of *E. coli* – genetic structure and role in pathogenicity. In *Bacteria, Complement and the Phagocytic Cell*, NATO ASI series H24, pp. 221–229. Edited by F. C. Cabello & C. Pruzzo. Berlin: Springer-Verlag.
- HAREL, J., FORGET, C., SAINT-AMAND, J., DAIGLE, F., DUBREUIL, D., JACQUES, M. & FAIRBROTHER, J. M. (1992). Molecular cloning of a determinant coding for fimbrial antigen F165, a Prs-like fimbrial antigen from porcine septicemic *Escherichia coli*. *Journal of General Microbiology* **138**, 1495–1502.
- HAREL, J., DAIGLE, F., MAITI, S., DESAUTELS, C., LABIGNE, A. & FAIRBROTHER, J. M. (1991a). Occurrence of *pap*-, *sfa*-, and *afa*-related sequences among F165-positive *Escherichia coli* from diseased animals. *FEMS Microbiology Letters* **82**, 177–182.
- HAREL, J., LAPOINTE, H., FALLARA, A., LORTIE, L.-A., BIGRAS-POULIN, M., LARIVIÈRE, S. & FAIRBROTHER, J. M. (1991b). Detection of genes for fimbrial antigens and enterotoxins associated with *Escherichia coli* serogroups isolated from pigs with diarrhea. *Journal of Clinical Microbiology* **29**, 745–752.
- KARR, J. F., NOWICKI, B., TRUONG, L. D., HULL, R. A. & HULL, S. I. (1989). Purified P fimbriae from two cloned gene clusters of a single pyelonephritogenic strain adhere to unique structures in the human kidney. *Infection and Immunity* **57**, 3594–3600.
- KORHONEN, T. K., VALTONEN, M. V., PARKKINEN, J., VAISANEN-RHEN, V., FINNE, J., ØRSKOV, F., SVENSON, S. B. & MAKELA, P. H. (1985). Serotypes, hemolysin production, and receptor recognition of *Escherichia coli* strains associated with neonatal sepsis and meningitis. *Infection and Immunity* **48**, 486–491.
- LINDAHL, M., FARIS, A., WADSTRÖM, T. & HJERTÉN, S. (1981). A new test based on salting out to measure relative hydrophobicity of bacterial cells. *Biochimica et Biophysica Acta* **677**, 471–476.
- LUND, B., MARKLUND, B.-I., STRÖMBERG, N., LINDBERG, F., KARLSSON, K.-A. & NORMARK, S. (1988). Uropathogenic *Escherichia coli* express serologically identical pili with different receptor binding specificities. *Molecular Microbiology* **2**, 255–263.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MANOIL, C. & BECKWITH, J. (1985). *TnphoA*: a transposon probe for protein export signals. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8129–8133.
- MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *Journal of Molecular Biology* **3**, 208–218.
- MILLER, I., MASKELL, D., HORMAECHÉ, C., JOHNSON, K., PICKARD, D. & DOUGAN, G. (1989). Isolation of orally attenuated *Salmonella typhimurium* following *TnphoA* mutagenesis. *Infection and Immunity* **57**, 2758–2763.
- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MILLER, V. L. & MEKALANOS, J. J. (1988). A novel suicide vector and its use in construction of an insertion mutation: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *Journal of Bacteriology* **170**, 2575–2583.

- MORRIS, J. A., THORNS, C. J., SCOTT, A. C. & SOJKA, W. J. (1982). Virulence properties associated with the *Vir* plasmid: a transmissible pathogenic characteristic associated with strains of invasive *Escherichia coli*. *Journal of General Microbiology* **128**, 2097–2103.
- ØRSKOV, I. & ØRSKOV, F. (1985). *Escherichia coli* in extraintestinal infections. *Journal of Hygiene* **95**, 551–575.
- PLUSCHKE, G., MAYDEN, J., ACHTMAN, M. & LEVINE, R. P. (1983). Role of the capsule and the O antigen in resistance of O18:K1 *Escherichia coli* to complement mediated killing. *Infection and Immunity* **42**, 907–913.
- REED, L. J. & MUENCH, H. (1938). A simple method of estimation for fifty per cent endpoints. *American Journal of Hygiene* **27**, 493–497.
- SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.
- TAYLOR, P. W. (1988). The mode of C5b-9 attack on susceptible Gram-negative bacteria. In *Bacteria, Complement, and the Phagocytic Cell*, NATO ASI series H24, pp. 129–138. Edited by C. F. Cabello & C. Pruzzo. Berlin: Springer-Verlag.
- TAYLOR, P. W. & KROLL, H. P. (1983). Killing of an encapsulated strain of *Escherichia coli* by human serum. *Infection and Immunity* **39**, 122–131.
- TAYLOR, R. K., MILLER, V. L., FURLONG, D. B. & MEKALANOS, J. J. (1987). Use of *phoA* fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proceedings of the National Academy of Sciences of the United States of America* **84**, 2833–2837.
- TAYLOR, R. K., SHAW, C., PETERSON, K., SPEARS, P. & MEKALANOS, J. J. (1988). Safe, live *Vibrio cholerae* vaccines? *Vaccine* **6**, 151–154.
- TAYLOR, R. K., MANOIL, C. & MEKALANOS, J. J. (1989). Broad-host-range vectors for delivery of *TnphoA*: use in genetic analysis of secreted determinants of *Vibrio cholerae*. *Journal of Bacteriology* **171**, 1870–1878.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.