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

J. HAREL,* C. FORGET, M. NGELEKA, M. JACQUES and J. M. FAIRBROTHER

Department of Pathology and Microbiology, Faculty of Veterinary Medicine, University of Montreal, Saint-Hyacinthe, Québec, Canada J2S 7C6

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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, cytotoxic necrosis toxin) (Morris et al., 1982; Korhoncn 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 F165_1 and F165_2, 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 septicemia 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...
Tn5 IS50(\(::\)phoA) (TnphoA), 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 TnphoA 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 \(\text{phoA}\) gene product into the periplasmic space (Manoil & Beckwith, 1985). Because most bacterial virulence determinants are expressed at the cell surface, this technique selects for TnphoA insertions into such genes (Taylor et al., 1987, 1989; Finlay et al., 1988; Miller et al., 1989). Thus, using the TnphoA system, isogenic strains of a pathogenic F165-positive strain of \(\text{E. coli}\) 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 \(\text{E. coli}\) 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 \(\text{pap}\) and \(sfa\) operons (Harel et al., 1991a). \(\text{E. coli}\) strain SM10 possessing plasmid pRT733 carrying TnphoA 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 mutagenesis (Taylor et al., 1989). Thus, using the TnphoA system, isogenic strains of a pathogenic F165-positive strain of \(\text{E. coli}\) 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 \(\text{E. coli}\) septicaemia.

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**Mutagenesis.** Mutations were induced by random insertion of the TnphoA sequence into chromosomal or plasmid DNA of \(\text{E. coli}\) strain 5131. The mutagenesis was accomplished by using the plasmid pRT733 which carries the TnphoA insertion in donor \(\text{E. coli}\) strain SM10 (\(\lambda\) 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 \(\lambda\) pir transducing phage, but it can be mobilized from strain SM10 (\(\lambda\) pir) into strain 5131 during mating. Fresh colonies of donor strain SM10 carrying the vector pRT733 were mated with recipient strain 5131 (Sm\(^-\)Ap\(^+\)) in LB plates and then incubated at 37 \(^\circ\text{C}\) for 16 h. After dilution, the mating mix was then spread onto agar containing streptomycin and kanamycin. Therefore, selection for Km\(^+\) and Sm\(^+\) resulted in the transposition of TnphoA into the chromosome or plasmid of the Sm\(^+\) recipient. The indicator XP (5-bromo-4-chloro-3-indolyl phosphate) was included in the agar to detect those transpositions that resulted in active phoA gene fusions in the recipient. Of approximately 2000 Kmr Sm\(^+\) 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 \(\times\) SSC (1 \(\times\) SSC is 0.15 M-NaCl + 0.015 M-sodium citrate) twice for 5 min at room temperature each time, then in 2 \(\times\) SSC with 0.5% SDS twice for 30 min each time at 65 \(^\circ\text{C}\), and finally in 0.1 \(\times\) SSC twice for 5 min each time at room temperature. Fimbrial probes representing the full F165, \(\text{Operon} \) (BamHI fragment from pCJ7, encoding the F165, \(\text{Operon} \) (Harel et al., 1992) and sfa \(\text{Operon} \) (EcoRV 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 \((\alpha\text{-}^{32}\text{P})\text{dCTP}\) 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 OD\(_{600}\) 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 (Contrepois 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 \(\text{E. coli}\) strain 5131 and from TnphoA 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 \(\text{phoA}\) 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 \(^\circ\text{C}\) 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% (w/v) Tween 20 (TTS), plus 2.5% (w/v) skim milk. Filters were then incubated for 1 h at 25 \(^\circ\text{C}\) 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 (AphoA), 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-Na₂HPO₄, 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 10⁶ to 10⁹ 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 lusions

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 K. smt 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

<p>| Table 1. Characteristics and virulence in chickens of TnphoA mutants of E. coli strain 5131 |</p>
<table>
<thead>
<tr>
<th>Mutant no.</th>
<th>MRHA*</th>
<th>F165 antigen†</th>
<th>Hydrophobicity value (M)‡</th>
<th>Survival in serum (%§)</th>
<th>log₁₀ LD₅₀¶</th>
<th>Virulence</th>
</tr>
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<tbody>
<tr>
<td>Wild-type</td>
<td>5131</td>
<td>+</td>
<td>+</td>
<td>0.125</td>
<td>227</td>
<td>6-8</td>
</tr>
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<td>Mutants</td>
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<td></td>
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<td>Class I</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4</td>
<td>117</td>
<td>6-8</td>
</tr>
<tr>
<td>M43</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>125</td>
<td>6-8</td>
</tr>
<tr>
<td>M48</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>138</td>
<td>6-7</td>
</tr>
<tr>
<td>15</td>
<td>+/−</td>
<td>+/−</td>
<td>4</td>
<td>10</td>
<td>7-2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>Class II</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>+</td>
<td>ND</td>
<td>4-7</td>
<td>7-3</td>
<td>Intermediate</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>+</td>
<td>0-25</td>
<td>29</td>
<td>7-9</td>
<td>Intermediate</td>
</tr>
<tr>
<td>17</td>
<td>-</td>
<td>+</td>
<td>0-5</td>
<td>5-8</td>
<td>7-8</td>
<td>Intermediate</td>
</tr>
<tr>
<td>18</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>1-2</td>
<td>7-2</td>
<td>Intermediate</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>0-5</td>
<td>0-8</td>
<td>8-9</td>
<td>Avirulent</td>
</tr>
</tbody>
</table>

* 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. Southern blot analysis of genomic DNA from 10 mutant strains digested with BamHI/EcoRV. The probe was a 1.9 kb HindIII-BamHI TnphoA-derived fragment that in BamHI/EcoRV 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 S131; 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. Southern blot analysis of genomic DNA from 10 mutant strains digested with BamHI/EcoRV. The probe was a 9 kb BamHI fragment derived from plasmid pCJ7, a prs-like operon, coding for F165+. Lane 1, wild-type strain S131; 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-).

eythrocytes, 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 EcoRV does not cut within transposon TnphoA, Southern hybridization of EcoRV-digested chromosomal DNA probed with an internal HindIII 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 EcoRV fragments ranging from 9 to 20 kb [including the inserted TnphoA (7-6 kb)]. One MRHA-F165- mutant, mutant 15, possessed two EcoRV 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 BamHI, which cuts once in TnphoA (data not shown), and with BamHI and EcoRV (Fig. 1). The DNA probe was a 1.9 kb HindIII-BamHI TnphoA-derived fragment that with BamHI/EcoRV 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 BamHI/EcoRV, 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 4.4–5.5 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.

<table>
<thead>
<tr>
<th>Strain or mutant*</th>
<th>MD-1 37°C/18°C</th>
<th>Ratio of PhoA activity at 37°C/18°C</th>
<th>MD-1 + 5 mm-alanine</th>
<th>Ratio of PhoA activity of cells grown on MD-1/MD-1 + alanine</th>
<th>MD-1 + 10 g glucose l⁻¹</th>
<th>Ratio of PhoA activity of cells grown on MD-1/MD-1 + glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>5131</td>
<td>0.18/0.54</td>
<td>0.3</td>
<td>0.4</td>
<td>0.45</td>
<td>0.18</td>
<td>1.0</td>
</tr>
<tr>
<td>Class I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M9 (MRHA⁻ F165⁻)</td>
<td>40.31/4.82</td>
<td>8.36</td>
<td>17.2</td>
<td>2.3</td>
<td>29.1</td>
<td>1.4</td>
</tr>
<tr>
<td>M43 (MRHA⁻ F165⁻)</td>
<td>28.09/9.11</td>
<td>3.95</td>
<td>10.5</td>
<td>2.7</td>
<td>12.9</td>
<td>2.2</td>
</tr>
<tr>
<td>M48 (MRHA⁻ F165⁻)</td>
<td>25.82/4.39</td>
<td>5.88</td>
<td>14.1</td>
<td>1.8</td>
<td>10.1</td>
<td>2.6</td>
</tr>
<tr>
<td>15 (MRHA⁻ F165⁻)</td>
<td>20.90/0.7</td>
<td>29.9</td>
<td>11.1</td>
<td>1.9</td>
<td>6.7</td>
<td>3.1</td>
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<td>Class II</td>
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<td></td>
</tr>
<tr>
<td>6 (MRHA⁺ F165⁺)</td>
<td>10.61/0.0</td>
<td>5.3</td>
<td>5.5</td>
<td>1.9</td>
<td>3.16</td>
<td>3.4</td>
</tr>
<tr>
<td>9 (MRHA⁻ F165⁺)</td>
<td>10.91/3.4</td>
<td>8.4</td>
<td>5.9</td>
<td>1.8</td>
<td>6.2</td>
<td>3.75</td>
</tr>
<tr>
<td>17 (MRHA⁻ F165⁺)</td>
<td>10.12/0.4</td>
<td>4.2</td>
<td>4.8</td>
<td>2.1</td>
<td>4.1</td>
<td>2.4</td>
</tr>
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</table>

* 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/BamHI-digested chromosomal DNA from E. coli strain 5131 and the class I and class II mutants was done using probes representing the full F165₁ operon (BamHI fragment from pC7, encoding the F165₁ operon) (Harel et al., 1992) and the sfa operon (EcoRV fragment from pANN801-13) (Hacker et al., 1985). The F165₁ operon shows a BamHI/EcoRV restriction map similar to theprs 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 F165₁ mutants. The blots demonstrated that TnphoA insertions in the class I and class II mutants were in the F165₁ operon, since the distribution of some bands hybridizing to the F165₁ probe had changed with respect to those of strain 5131 (Fig. 2). Moreover, the disappearance of F165₁-specific bands and the pattern of bands corresponding to the F165₁, sequences adjacent to TnphoA indicated that TnphoA insertions in class I strains were within the 5' end region (genes AHC) of the F165₁ 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 F165₁ 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+); lane 2, mutant 9 (MRHA+); lane 3, mutant M9 (MRHA-); 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 I), 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. 4a). In contrast, class I (MRHA-F165−) mutants were relatively non-hydrophobic (the

![Fig. 4](image)

Fig. 4. Electron micrographs after colloidal gold immunolabelling using rabbit anti-F165 serum of (a) *E. coli* strain 5131, (b) mutant 6 (MRHA+), and (c) mutant M9 (MRHA-). 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 log_{10} LD_{50} 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., 1988). 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 F165, 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 F165, produced by strain 5131. The TnphoA insertions of mutants of class I and class II were located in the F165, 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 bacterialcidal 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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