Complementation of Mutations in *Escherichia coli* and *Bordetella pertussis* by *B. pertussis* DNA Cloned in a Broad-host-range Cosmid Vector

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A gene library of *Bordetella pertussis* DNA was constructed in *Escherichia coli* using the broad-host-range cosmid vector pLAFR1. The average insert size was 24.9 kb. From 500 members of the gene library, clones were identified which complemented *trpE*, *glnA* and Thr⁻ mutations in *E. coli* but none which complemented *trpD*, *trpC*, *trpB*, *trpA*, *proA* or Leu⁻ mutations. Four clones were identified which complemented *trpE* in *E. coli*. Anthranilate synthase activity was detected in a *trpE* strain only when it harboured a plasmid from one of these clones; activity was repressed when tryptophan was included in the growth medium. Two clones were identified which complemented *glnA* of *E. coli*. A recombinant plasmid from one of these clones also restored some of the nitrogen acquisition functions of *glnG* and *glnL* in *E. coli*. Expression of several *B. pertussis* virulence-associated products (haemolysin, heat-labile toxin, adenylate cyclase, filamentous haemagglutinin, and the cell-envelope polypeptide of *M_r* 30000) was not detected in 500 independent clones. However, by transferring the recombinant plasmids to a mutant of *B. pertussis* deficient in haemolysin and adenylate cyclase, a plasmid was identified which restored both these activities.

INTRODUCTION

Bordetella pertussis is a pathogen which is difficult to study *in vitro* because of its sensitivity to inhibitory substances in conventional media (Parker, 1976), and its tendency to undergo antigenic variation with continual subculture (Leslie & Gardner, 1931). Several virulence-related components have been described: these include pertussis toxin (or pertussigen), heat-labile toxin (HLT) (Wardlaw & Parton, 1983), an adenylate cyclase toxin (Hewlett *et al.*, 1985), filamentous haemagglutinin (FHA) (Sato *et al.*, 1979), two major cell-envelope polypeptides of *M_r* 28000 and 30000 (Wardlaw & Parton, 1979) and cell-surface heat-labile agglutinogens (Preston, 1976). There has been a great deal of recent interest in these factors with a view to developing a defined acellular vaccine with greater efficacy and lower toxicity than the present whole-cell vaccine (Robinson *et al.*, 1985). However, little still is known of the role of these components in the pathogenesis of pertussis.

The advent of recombinant DNA technology has facilitated the study of pathogens which are difficult to grow and manipulate in the laboratory. However, initial attempts by Weiss & Falkow (1983) and Dr C. Duggelby (personal communication) to identify *B. pertussis* virulence determinants in gene libraries of the pathogen in *Escherichia coli* were unsuccessful. This may have been due to the apparent requirement for a trans-acting regulatory factor, identified by transposon mutagenesis (Weiss *et al.*, 1983), for the expression of several virulence determinants in *B. pertussis*. However, Shareck & Cameron (1984) reported the cloning and expression of the two virulence-associated cell-envelope polypeptides of *B. pertussis* in *E. coli*. These workers suggested that they may have cloned the trans-acting regulatory factor on the same insert.

Abbreviations: TE, Tris/HCl-EDTA buffer; HLT, heat-labile toxin; FHA, filamentous haemagglutinin; AS, anthranilate synthase; GS, glutamine synthetase.

We have constructed a gene library of *B. pertussis* in a broad-host-range cosmid vector, pLAFR1, with the rationale that if expression of *B. pertussis* virulence determinants in *E. coli* was not obtained, the gene library could be transferred to a suitable *Bordetella* host. The cosmid, pLAFR1, is a derivative of the broad-host-range vector pRK290 (Friedman *et al.*, 1982). Like pRK290, pLAFR1 is not self-transmissible, but can be conjugated into Gram-negative recipients in the presence of a helper plasmid, such as pRK2013, which contains the RK2 *tra* genes. Here we report the complementation of *E. coli* auxotrophs by our gene library. Although we were unable to detect expression of several *B. pertussis* virulence determinants in *E. coli*, a clone was identified which complemented adenylate cyclase and haemolysin-deficient mutants of *B. pertussis*.

METHODS

Bacterial strains and media. The bacteria used are listed in Table 1. *E. coli* strains were grown with shaking in nutrient broth (Oxoid). The solid and liquid minimal medium used, except where otherwise stated, was that described by Clowes & Hayes (1968). Depending on the phenotype of the strain, minimal medium was supplemented with amino acids at 20 µg ml⁻¹ (final concentration) and thiamin at 2 µg ml⁻¹ (final concentration). Other media used were M9 minimal medium (Maniatis *et al.*, 1982) and CL medium (Imaizumi *et al.*, 1983). *B. pertussis* was grown on Bordet–Gengou (BG) medium (Gibco-BRL) containing 20% (v/v) defibrinated horse blood (Gibco-BRL) for 72 h at 35 °C in a moist atmosphere. A loopful of these cultures was used to inoculate 50 ml of a defined (Stainer and Scholte) medium (Stainer & Scholte, 1971) in 250 ml flasks. The flasks were incubated in an orbital incubator at 80 r.p.m. at 37 °C. For selective and maintenance purposes, antibiotics (Sigma) were present in media at the following final concentrations: tetracycline, 10 µg ml⁻¹; streptomycin, 200 µg ml⁻¹; and kanamycin, 20 µg ml⁻¹.

Isolation of DNA. Plasmid DNA was isolated by the method of Birnboim & Doly (1979). Plasmid for the construction of the gene library was further purified by centrifugation to equilibrium in a caesium chloride–ethidium bromide gradient as described by Maniatis *et al.* (1982). *B. pertussis* chromosomal DNA was prepared by a method based on that of Hull *et al.* (1981). Briefly, a 50 ml culture grown for 48 h was harvested by centrifugation and washed once in 1 mM-EDTA, 10 mM-Tris/HCl, pH 8.0 (TE) with 100 mM-NaCl. Washed cells were resuspended in 20 ml 25% (w/v) sucrose in 50 mM-Tris/HCl, 1 mM-EDTA pH 8.0 on ice. Lysozyme (Sigma) was added (50 µl 40 mg lysozyme ml⁻¹ in TE). After 5 min at room temperature, 10 µl proteinase K (Gibco-BRL) (20 mg ml⁻¹), 100 µl RNase A (Sigma) (10 mg ml⁻¹ heated before use at 100 °C for 15 min) and 400 µl 0.5 M-EDTA were added. Cells were lysed by the addition of 250 µl 10% (w/v) sodium *N*-lauroylsarcosinate, and were allowed to rest on ice until lysis was complete. The lysate was incubated at 42 °C for 60 min and extracted three times with equal volumes of 1:1 (v/v) phenol (TE-saturated)/chloroform followed by one extraction with chloroform alone. To the aqueous layers, 1/10 vol. 3 M-sodium acetate was added followed by 2 vols ethanol. The precipitated DNA was wound off and slowly dissolved in 1.5 ml TE, and dialysed at 4 °C in the same buffer with three changes over 3 d.

Construction of the gene library. Ligations, digestions with restriction enzymes and treatment with alkaline phosphatase were done according to the manufacturer's instructions. *B. pertussis* DNA was partially digested for 1 h with *EcoRI* (Gibco-BRL) using a doubling dilution series of the enzyme. Digestions were terminated by adding EDTA to a final concentration of 20 mM and heating to 68 °C for 10 min. Those digests which generated fragment lengths of 15–50 kb (determined by electrophoresis on a 0.3%, w/v, agarose gel) were pooled, and loaded onto a 20–40% (w/v) sucrose gradient in 1 M-NaCl, 20 mM-Tris/HCl, 5 mM-EDTA, pH 8.0. After centrifugation at 50000 g at 20 °C for 24 h, fractions were collected and those with fragments in the 20–35 kb region were pooled and dialysed extensively in TE. The cosmid LAFR1 (21.6 kb) was completely digested with *EcoRI* (pLAFR1 has a unique *EcoRI* site), and then treated with calf intestinal alkaline phosphatase (Boehringer-Mannheim). Chromosomal DNA and pLAFR1 were ligated, with cosmid present in a fivefold higher molar concentration, using Ligaid (P & S Biochemicals Ltd, Liverpool, UK), and packaged *in vitro* as described by Maniatis *et al.* (1982). *E. coli* DH1 was infected with the packaged DNA as reported by Ish-Horowicz & Burke (1981). Tetracycline-resistant colonies (pLAFR1 confers tetracycline resistance) were picked and subcultured in nutrient broth and stored at –20 °C with an equal volume of glycerol.

Nick-translation and hybridization. The method of Rigby *et al.* (1977) was used to nick-translate *B. pertussis* DNA. Plasmid preparations from recombinant clones were spotted onto nitrocellulose and allowed to hybridize with ³²P-labelled *B. pertussis* DNA according to a procedure described by Maniatis *et al.* (1982).

Gel electrophoresis. DNA molecules were separated on 0.7% or 0.3% (w/v) agarose (Sigma type I or Seakem HGT) gels in a running buffer containing 90 mM-Tris base, 90 mM-boric acid, 1 mM-EDTA, pH 8.3. *M_r* values were estimated by comparison with an *EcoRI* digest of λ DNA.

Mating methods. For *E. coli* to *E. coli* conjugation experiments, overnight cultures of the recipient, the donor and the 'helper strain' [HB101(pRK2013)] were mixed together in equal volumes, spread on nutrient agar plates and

Table 1. Strains of *E. coli* and *B. pertussis*

Strain	Genotype/properties	Source or reference
<i>E. coli</i> DH1	<i>recA1 endA1 gyrA96 Thi⁻hsdR17 supE44</i>	Maniatis <i>et al.</i> (1982)
<i>E. coli</i> HB101	<i>hsdS20 recA13 Ara⁻proA2 lacY1 galK2 rpsL20</i>	
	Xyl Mlt ⁻ <i>supE44</i>	
<i>E. coli</i> HB101(pLAFR1)		Friedman <i>et al.</i> (1982)
<i>E. coli</i> HB101(pRK2013)		
<i>E. coli</i> JC3272	Lys ⁻ Trp ⁻ His ⁻ Strp ^r	Achtman <i>et al.</i> (1971)
<i>E. coli</i> Th16*	Thi ⁻ <i>endA hsr lacU169 glnA::Tn5</i> (contains <i>K. aerogenes</i> Hut operon with <i>hutC</i>)	Dr B. Magasanik, Massachusetts Institute of Technology, Cambridge, Massachusetts, USA
<i>E. coli</i> YMC11*	Thi ⁻ <i>endA hsr lacU169 ΔglnG-glnA</i> (contains <i>K. aerogenes</i> Hut operon with <i>hutC</i>)	
<i>E. coli</i> P678F ⁻	Thr ⁻ Leu ⁻ Thi ⁻ Lac ⁻ Strp ^r	Clowes & Hayes (1968)
<i>E. coli</i> KF51*	<i>trpE</i>	
<i>E. coli</i> KF52*	<i>trpA</i>	
<i>E. coli</i> KF54*	<i>trpB</i>	NCIB, Torrey Research Station, Aberdeen, UK
<i>E. coli</i> NCIB 10766*	<i>trpC</i>	
<i>E. coli</i> NCIB 10767*	<i>trpD</i>	
<i>E. coli</i> CA8306*	<i>Δcyd Thi⁻</i>	Kiely & O'Gara (1983)
<i>B. pertussis</i> TAB I		Brownlie <i>et al.</i> (1985)
<i>B. pertussis</i> 347	A Tn5 mutant deficient in FHA, adenylate cyclase, haemolysin, HLT and pertussis toxin	Weiss <i>et al.</i> (1983)
<i>B. pertussis</i> 348	A Tn5 mutant deficient in haemolysin and adenylate cyclase	
<i>B. pertussis</i> 349	A Tn5 mutant deficient in haemolysin	

* Spontaneous streptomycin resistant mutants were isolated from these strains and used in conjugation experiments.

incubated overnight at 37 °C. Subsequent growth was diluted in saline (0.85% NaCl), and dilutions were plated onto appropriate selective medium. When individual clones were screened for their ability to complement auxotrophs, static overnight cultures in nutrient broth of the recipient and HB101(pRK2013) were mixed together in equal volumes and added (an equal volume) to the clones grown overnight in wells of microtitre plates containing nutrient broth. After mixing, a sample from each well was spotted onto nutrient agar and incubated overnight at 37 °C. Growth from these plates was subsequently spotted onto selective minimal medium. The selective medium contained tetracycline and streptomycin for selection of the plasmid and recipient respectively; for selection of plasmid which would complement the auxotrophic marker of the recipient, the appropriate amino acid was not included in the minimal medium. For *E. coli* to *B. pertussis* conjugation experiments, the same procedure was followed except that *B. pertussis* was grown in Stainer and Scholte medium before mixing with *E. coli*. Mixtures were spotted onto BG plates, and the subsequent growth was transferred to selective BG plates containing tetracycline, kanamycin (the *B. pertussis* recipients used were resistant to kanamycin) and 5×10^7 p.f.u. T1 phage ml⁻¹ (as an added selection against *E. coli*).

Screening procedures for *B. pertussis* virulence determinants. FHA was screened for by an enzyme-linked immunosorbent assay. Briefly, colonies were grown on CL plates overnight and transferred to prewetted nitrocellulose filters (Schleicher and Schuell; BA85, 0.45 μm). Filters were placed above a dish of chloroform for 10 min, to lyse the cells, and incubated overnight at 4 °C in 5% (w/v) BSA in PBS (8.0 g NaCl l⁻¹, 0.2 g KCl l⁻¹, 1.15 g Na₂HPO₄ l⁻¹, 0.2 g KH₂PO₄ l⁻¹). After three 15 min washes in PBS, the filters were incubated with a 1/10 dilution (in PBS with 1%, w/v, BSA) of a cocktail of mouse monoclonal anti-FHA antisera (kindly supplied by Dr L. A. E. Ashworth, PHLS Centre for Applied Microbiology and Research, Porton Down, UK) with gentle shaking for 2 h at room temperature. The filters were washed as before and incubated with a 1/3000 dilution (in PBS with 1%, w/v, BSA) of anti-mouse IgG horse-radish peroxidase enzyme conjugate (Scottish Antibody Production Unit, Law Hospital, Carlisle, UK) with gentle shaking for 2 h at room temperature. After washing the filters three times as before, the filters were incubated with substrate [2 mg 3-amino-9-ethylcarbazole ml⁻¹ (Sigma), 0.024%, v/v, H₂O₂ in 50 mM-sodium acetate, pH 5.0]. The reaction was terminated after 30 min by placing the filters in distilled water.

The cell-envelope polypeptide of *M. 30000* was screened for by the same procedure except that a 1/100 dilution of mouse monoclonal antiserum specific to this polypeptide (kindly supplied by Dr J. B. Hertz, Department of Clinical Microbiology, Hvidovre Hospital, Copenhagen, Denmark) was used in place of anti-FHA.

To screen for HLT, clones were grown overnight in CL medium in wells of microtitre plates and injected subcutaneously into three-week-old mice [four cultures (50 µl each) per mouse]. Mice were examined the next day for a haemorrhagic reaction (Parton, 1986).

Haemolytic activity was screened for by spotting clones onto BG sandwich plates. Grown cells were lysed either by leaving the plates at room temperature for 5 d or by treatment with chloroform vapour for 10 min. After 24 h incubation, both at room temperature and at 4 °C, clones were examined for a zone of haemolysis.

To screen for adenylate cyclase, the recombinant plasmids were transferred from *E. coli* DH1 to *E. coli* CA8306 (Δcya) using the method described above. Complementation of the *cya* mutation was screened for by selection on minimal medium plates with tetracycline, streptomycin and with the glucose replaced by either lactose or maltose.

Enzyme assays. Bacteria were grown to mid-exponential phase in litre volumes in minimal medium for *E. coli* strains, or Stainer and Scholte medium for *B. pertussis* strains. Cells were harvested by centrifugation, washed once in 50 mM-potassium phosphate, 1 mM- β -mercaptoethanol, pH 7.5, resuspended in about 2.0 ml of the same buffer and disrupted using an MSE ultrasonic disintegrator for three 40 s intervals with intervening cooling. Debris was removed by centrifugation at 20000 g for 30 min at 4 °C. Supernates were assayed for anthranilate synthase (AS) activity, histidase activity, glutamine synthetase (GS) activity (using the γ -glutamyltransferase assay) and adenylate cyclase activity by the methods described by Baskerville & Twarog (1972), Coote (1974), Bender *et al.* (1977) and Brownlie *et al.* (1985), respectively. Protein was estimated by the method of Herbert *et al.* (1969) with BSA as standard.

RESULTS AND DISCUSSION

Construction of gene library of B. pertussis

The gene library of *B. pertussis* was constructed in the cosmid vector pLAFR1 as described in Methods. The efficiency of the procedure was about 20000 tetracycline resistant colonies per µg of *B. pertussis* DNA packaged. From ten clones, the average insert size was calculated to be 24.9 kb (SD 4.35). Hybridization studies with plasmid preparations and nick-translated ^{32}P -labelled *B. pertussis* DNA confirmed that the insert, for the ten clones, was *B. pertussis* DNA (no hybridization was observed for pLAFR1 without insert). We subcultured 500 tetracycline-resistant recombinants and stored these frozen with added glycerol. From the equation derived by Clarke & Carbon (1976) and assuming *B. pertussis* to have a genome of similar size to that of *E. coli* (4100 kb), the gene library was calculated to be >95% complete.

Complementation of E. coli auxotrophs

To determine whether the reported inability of *E. coli* to express *B. pertussis* DNA was a general phenomenon or confined to virulence determinants, the gene library was tested for its ability to relieve auxotrophic phenotypes in *E. coli*. Initially, the gene library was pooled into groups of 50 clones for convenience and these were conjugated *en masse* into the *E. coli* strain JC3272 (His⁻ Trp⁻ Lys⁻). Three of the clone pools complemented the Trp⁻ phenotype but none complemented the His⁻ phenotype (a high reversion rate of the recipient to Lys⁺ made this an unsuitable marker to score for complementation). For each of these three pools, the rate of Trp⁻ complementation was roughly 1/50 the rate of transfer of recombinant pLAFR1 (i.e. the rate of tetracycline resistance transfer). Although this suggested that one clone, out of the 50 in each pool, was responsible for the observed complementation, it was possible that the clones were not all growing at the same rate, and that some may have been diluted out of recognition. Therefore, subsequent crosses were done using clones individually grown in microtitre plates.

Using this method, four clones (strain DH1 with either pLAFR1018, pLAFR1252, pLAFR1390 or pLAFR1397) were identified which complemented the *trpE* mutation of *E. coli* KF51. However, none were identified which complemented the *trpA*, *trpB*, *trpC* or *trpD* mutations of *E. coli* (strains KF52, KF54, NCIB 10766 and NCIB 10767, respectively). The four clones which complemented *trpE* could be traced back to the three pools which complemented the Trp⁻ phenotype of strain JC3272 in earlier experiments. As none of these clones complemented any of the other Trp⁻ mutations, strain JC3272 was presumed to be *trpE*. The reason why only one of the five genes required for tryptophan synthesis in *E. coli* was complemented by the *B. pertussis* gene library is not known. It could be due to physiological incompatibilities between the two organisms, either in the tryptophan synthetic pathway or in the manner in which expression of the responsible genes is regulated. The former seems unlikely

Table 2. Anthranilate synthase activities in extracts of *B. pertussis* and *E. coli*

E. coli strains were grown in minimal medium and *B. pertussis* was grown in Stainer and Scholte medium. Activities are expressed as pmol anthranilate formed (mg protein)⁻¹ min⁻¹ $\pm$ SD. The number of determinations are given in parentheses. Tryptophan, when present in the growth medium, was at a final concentration of 20 μ g ml⁻¹.

Bacterial strain	Tryptophan present (+) or absent (-) in growth medium	AS activity
<i>E. coli</i> DH1	-	0.84 $\pm$ 0.26 (4)
<i>E. coli</i> JC3272	+	<0.05 (2)
<i>E. coli</i> JC3272(pLAFR1018)	-	0.84 $\pm$ 0.33 (3)
<i>E. coli</i> JC3272(pLAFR1018)	+	0.08 $\pm$ 0.03 (3)
<i>B. pertussis</i> TAB I	-	4.82 $\pm$ 0.22 (4)
<i>B. pertussis</i> TAB I	+	0.54 $\pm$ 0.28 (2)

in that the tryptophan synthetic pathway is unusually well-conserved for the broad range of organisms studied; on the other hand, its genetic organization and regulation is quite diverse (Crawford, 1975). Alternative explanations are that modification of certain regions of *B. pertussis* DNA may occur in *E. coli* preventing its expression, or simply that the genes are not represented in the library.

Plasmid preparations of the four clones were digested with *Eco*RI. Two bands of approximately 20 kb and 2.8 kb were common to the four clones. Plasmids pLAFR1018, pLAFR1252 and pLAFR1397 had an additional small fragment of about 2.4 kb, and pLAFR1252 also had a fragment of about 4.5 kb. Plasmid pLAFR1390, on the other hand, had an additional fragment of about 5.3 kb. From this it can be concluded that anthranilate synthase, (the product of *trpE*) is encoded somewhere on the two common fragments which are flanked on either side by an *Eco*RI fragment of 5.3 kb and two *Eco*RI fragments of 2.4 and 4.5 kb. That none of these clones complemented *trpA*, *trpB*, *trpC*, or *trpD* would suggest that the Trp biosynthetic genes of *B. pertussis* are not clustered in a single operon; however, the inability to detect these genes elsewhere in the gene library by complementation does not substantiate this.

Glutamine-dependent AS activity was determined in cell-free extracts of *E. coli* JC3272 (*trpE*) harbouring the recombinant plasmid pLAFR1018 (Table 2), and was compared with that in *E. coli* DH1 and *B. pertussis* TAB I. When grown in the absence of tryptophan, pLAFR1018 restored AS activity of the *trpE* strain to a level comparable to that expressed by *E. coli* DH1, but not as high as that expressed by *B. pertussis* grown without tryptophan; this would suggest that expression was under the influence of the *E. coli* regulatory machinery. Expression of AS from pLAFR1018 was repressed about tenfold when the strain was grown in the presence of tryptophan; similarly AS was repressed about tenfold in *B. pertussis* when grown with tryptophan. Because of the large size of the insert in pLAFR1018, it is not known whether the observed repression is due to the Trp repressor of *E. coli*, or a repressor of *B. pertussis*. Alternatively, some other mechanism such as attenuation may be involved. Glutamine-dependent AS activity, for all organisms studied so far (Crawford, 1975), not only depends on the large *trpE* subunit but also on a smaller glutamine amido-transfer subunit which is one of the *trpD* products in *E. coli*. Whether or not a *B. pertussis* *trpE* product is acting in conjunction with an *E. coli* *trpD* product in *E. coli* JC3272(pLAFR1018) requires further investigation.

Other auxotrophic markers in *E. coli* which were complemented were an undefined Thr⁻ mutation in *E. coli* P678F⁻, by pLAFR1315, and *glnA* (GS) of *E. coli* TH16 by either pLAFR1132 or pLAFR1109. These were identified by the ability of the strains P678F⁻ and TH16 to grow on minimal medium without threonine and glutamine, respectively. *Eco*RI digests of plasmid preparations of these clones indicated that the insert consisted of the following fragments (size in kb): pLAFR1315, 10.0, 8.6, 3.0, 2.5 and 2.3; pLAFR1109, 6.7, 4.6, 4.0, 3.8, 3.6 and 3.0; pLAFR1132, 6.7, 4.6, 4.0, 3.6, 3.0 and 2.3. With the exception of two fragments, the *Eco*RI digest profiles were the same for pLAFR1109 and pLAFR1132. Both of these plasmids, when transferred to *E. coli* YMC11, enabled this strain also to grow on minimal medium without glutamine.

Table 3. *Glutamine synthetase activity in extracts of E. coli TH16 and YMC11 with and without pLAFR1109*

E. coli strains were grown in M9 medium where the NH_4Cl was replaced by 15 mM-sodium glutamate together with glutamine at the concentration indicated. GS activity is expressed as $\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed (mg protein)}^{-1} \text{ min}^{-1}$. Results are the means of three determinations $\pm$ SD.

<i>E. coli</i> strain	Glutamine concn (mM)	GS activity
YMC11*	15	<0.001
TH16*	15	<0.001
YMC11(pLAFR1109)	15	0.37 $\pm$ 0.03
YMC11(pLAFR1109)	0.15	1.68 $\pm$ 0.17
TH16(pLAFR1109)	15	0.38 $\pm$ 0.02
TH16(pLAFR1109)	0.15	1.84 $\pm$ 0.14

* Strains without plasmid did not grow well with 0.15 mM-glutamine.

Table 4. *The ability of E. coli strains YMC11 and TH16 with and without pLAFR1109 to grow on minimal medium with various nitrogen sources*

Strains were inoculated onto M9 plates where the NH_4Cl was replaced by the nitrogen sources indicated. The relative level of growth (–, no growth; ++, moderate growth; + + + +, good growth) was determined after 48 h incubation at 37 °C.

Strain	NH_4Cl (20 mM)	Arginine (10 mM) + glutamine (0.5 mM)	Proline (15 mM) + glutamine (0.5 mM)	Glutamine (15 mM)	Glutamine (0.5 mM)
YMC11 ($\Delta\text{glnG-glnA}$)	–	–	–	+ + + +	–
YMC11(pLAFR1109)	+ + + +	+ + + +	+ + + +	+ + + +	+ +
TH16 ($\text{glnA}::\text{Tn5}$)	–	+ +	+ +	+ + + +	+ +
TH16(pLAFR1109)	+ + + +	+ + + +	+ + + +	+ + + +	+ +

GS activity was assayed in exponential phase *E. coli* TH16, *E. coli* YMC11 and in these two strains harbouring pLAFR1109 (Table 3). Activity could not be detected in either *E. coli* TH16 or *E. coli* YMC11, but significant levels were detected in strains harbouring pLAFR1109. Activity was approximately fivefold greater when strains harbouring the plasmid were grown under nitrogen-limiting conditions.

It has previously been shown that in *E. coli* the nitrogen regulatory genes (*glnL* and *glnG*, which are closely linked to *glnA*) control the level of arginine transport activity, the high affinity glutamine transport system and the activation of the *Klebsiella* Hut operon carried by strains TH16 and YMC11 (Tuli *et al.*, 1982). These functions enable enteric bacteria to utilize alternative nitrogen sources when NH_4^+ concentrations are low (Magasanik, 1982). The presence of analogous nitrogen regulatory genes on pLAFR1109 was assessed in a similar manner to the way in which these were characterized in cloned *E. coli* DNA (Tuli *et al.*, 1982) and in cloned *Thiobacillus ferrooxidans* DNA (Barros *et al.*, 1985). *E. coli* strains were assessed for their ability to grow on M9 medium with various nitrogen sources (Table 4). Strains YMC11 and TH16 were unable to grow with NH_4Cl as the sole nitrogen source due to lack of GS (*glnA*); when strains harboured pLAFR1109, however, they grew well with NH_4Cl as the sole nitrogen source. Strain YMC11, unlike strain TH16 which contains *glnL* and *glnG* expressed from the *glnL* promoter, was also unable to grow with arginine or proline and limiting glutamine, due to the lack of transport functions dependent on *glnL* and *glnG* activities. The plasmid pLAFR1109 enabled both strains to grow normally on these media indicating that the cloned *B. pertussis* DNA was able to complement the *glnL* and *glnG* functions in addition to the *glnA* functions of *E. coli*. Consistent with this was the further finding that histidase levels were increased when strain YMC11(pLAFR1109) was grown under nitrogen-limiting conditions (the *Klebsiella* Hut operon carried by this strain is *hutC* and therefore does not require histidine for induction). Cell extracts were prepared after growth of strain YMC11(pLAFR1109) in M9 medium where the NH_4Cl was replaced by 15 mM-sodium glutamate and either 0.15 mM- or 15 mM-glutamine. Means of

Fig. 1. Complementation of the haemolysin-deficient Tn5 mutant, *B. pertussis* 348, by pLAFR1138. Recombinant plasmids were transferred from *E. coli* DH1 to *B. pertussis* 348 as described in Methods. The arrow points to *B. pertussis* 348 harbouring pLAFR1138.

histidase activities [μmol urocanate formed (mg protein) $^{-1}$ min $^{-1}$ $\pm$ SD] from three determinations were as follows: 0.17 ± 0.01 after growth with 15 mM-glutamine, and 0.27 ± 0.03 after growth with 0.15 mM-glutamine. Strain YMC11 (without plasmid) grew very poorly under these nitrogen-limiting conditions (0.15 mM-glutamine), but it has previously been demonstrated that histidase levels do not increase when strain YMC11 and other *glnG glnL* strains are grown under the same nitrogen-limiting conditions (Tuli *et al.*, 1982; Barros *et al.*, 1985). These results suggest that *B. pertussis* GS itself, or other associated functions (within 25 kb), have the potential to regulate nitrogen acquisition in a manner similar to that in the enteric bacteria.

Other auxotrophic markers in *E. coli* which the gene library did not complement were *proA2* of strain HB101 and an undefined *Leu* $^{-}$ mutation of strain P678F $^{-}$. This may be for the reasons discussed above. The findings of this report, however, do indicate that it is possible to get expression of *B. pertussis* physiological functions in *E. coli*.

Screening for *B. pertussis* virulence determinants

The 500 *E. coli* clones were screened for expression of FHA, HLT, adenylate cyclase, haemolysin and the cell-envelope polypeptide of *M*, 30000 as described in Methods. Expression of these virulence determinants was not detected in the gene library in *E. coli*. These virulence determinants are lost concomitantly when *B. pertussis* is grown in medium where the NaCl is replaced by one of several salts or organic acids suggesting a common regulatory mechanism (Brownlie *et al.*, 1985). Weiss *et al.* (1983) isolated a single transposon insertion mutant of *B. pertussis* (strain 347) which lacked several virulence determinants. These workers suggested that a trans-acting function, which is required for expression of virulence determinants, was inactivated by transposon insertion in *B. pertussis* 347. A regulatory model was proposed (Weiss & Falkow, 1984) whereby expression of virulence factors is governed by the putative trans-acting factor in response to external stimuli. The observed lack of expression of *B. pertussis* virulence determinants in gene libraries, with *E. coli* as the host, is consistent with the proposed requirement for an additional *B. pertussis* function for expression of these determinants.

Recombinant plasmids were transferred from *E. coli* DH1 to *B. pertussis* 348 [a transposon mutant deficient in haemolysin and adenylate cyclase (Weiss *et al.*, 1983)] as described in Methods. A recombinant plasmid (pLAFR1138) was identified which complemented both the haemolytic activity (Fig. 1) and adenylate cyclase activity (results not shown) of *B. pertussis* 348. This same plasmid also restored the haemolytic activity of *B. pertussis* 349 (a transposon mutant deficient in haemolysin alone). This result supports the suggestion that the haemolysin and adenylate cyclase are closely linked, perhaps on the same operon (Weiss *et al.*, 1983). However, the haemolytic activity conferred by pLAFR1138 was not expressed when the plasmid was transferred to *B. pertussis* 347, confirming the requirement for a trans-acting factor for expression of haemolysin. Furthermore, no haemolytic activity or calmodulin-responsive adenylate cyclase activity was detected (results not shown) in *E. coli* DH1 harbouring pLAFR1138 [*B. pertussis* adenylate cyclase is unusual in its marked responsiveness to stimulation by calmodulin (Hewlett *et al.*, 1985)].

Future work will be aimed at subcloning and characterizing the adenylate cyclase and haemolysin determinants with a view to determining their role in pathogenesis.

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