Differentiation of phase I and variant strains of *Bordetella pertussis* on Congo red media

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Summary. The addition of Congo red, Trypan blue or haemin to the growth medium allowed the differentiation of phase-I and variant strains of *Bordetella pertussis*. Phase-I strains produced red (CR⁺), blue or dark brown colonies on a modified cyclodextrin solid medium containing Congo red, Trypan blue or haemin, respectively, whereas variant (Vir⁻ and phase IV) strains grew as pale (CR⁻) colonies. Spontaneous CR⁻ variants were isolated and characterised and had a phenotype like that of Vir⁻ or phenotypically modulated, C-mode strains in that they did not produce the haemolysin, haemagglutinin(s), histamine-sensitising factor (pertussis toxin), heat-labile toxin and two major envelope polypeptides associated with phase-I strains. Two such variants had reduced virulence for mice. CR⁺ strains, when grown on a high nicotinic acid medium to induce modulation, gave CR⁻ colonies. Thus the CR⁺ phenotype is a characteristic of phase-I *B. pertussis* and its expression appears to be controlled in a manner similar to that of other phase I-related factors. CR⁻ variants of *B. parapertussis* and *B. bronchiseptica* were also deficient in these factors. Four isolates of *B. avium* were CR⁻.

Introduction

With several bacterial species, it is possible to identify variant strains by their inability to bind certain dyes such as Congo red. Payne and Finkelstein (1977) found that strains of *Shigella*, *Vibrio cholerae*, *Escherichia coli* and *Neisseria meningitidis* grown on Congo red agar normally produced red (CR⁺) colonies. Variants that failed to absorb the dye produced colourless (CR⁻) colonies and had reduced virulence in a chick embryo model. Trypan blue, a bis-azo dye similar to Congo red, was absorbed by CR⁺ but not by CR⁻ strains.

Similar findings have been made with other species. CR⁺ and CR⁻ variants of a large number of strains of *Yersinia enterocolitica* and related species were tested for virulence in a mouse model and only CR⁺ strains were virulent (Prpic *et al.*, 1983, 1985). A correlation between Congo red binding and haemin binding by *Yersinia* spp. has also been noted (Surgalla and Beesley, 1969; Prpic *et al.*, 1985; Robins-Browne *et al.*, 1986). In *S. flexneri*, loss of the ability to bind Congo red was accompanied by loss of ability to invade epithelial cells in culture (Maurelli *et al.*, 1984). Congo red agar has also been used to select strains of the fish

pathogen *Aeromonas salmonicida* possessing a surface protein layer that is absolutely required for virulence (Ishiguro *et al.*, 1985). More recently, a direct correlation was noted between the ability of *E. coli* isolates to form CR⁺ colonies and their ability to cause septicæmic infections in chickens (Berkhoff and Vinal, 1986).

This simple visual test, the ability of bacterial colonies to absorb Congo red, Trypan blue or haemin from the growth medium, has been applied to strains of *Bordetella pertussis* and other *Bordetella* spp. for the differentiation of phase-I and variant strains. Phase-I (X-mode) strains of *B. pertussis* normally possess a characteristic range of properties, including production of haemolysin, heat-labile toxin, haemagglutinin(s), histamine-sensitising factor (pertussis toxin) and certain envelope polypeptides, and mouse-virulence, that are absent in genotypic (phase IV) and phenotypic (C-mode) variants (Robinson *et al.*, 1986). Weiss *et al.* (1983) introduced the terms “virulent phase” (Vir⁺ phenotype) and “avirulent phase” (Vir⁻ phenotype) as a concise description of strains with or without this usual range of (phase I-related) properties. It must be stressed however that such terminology does not necessarily reflect the behaviour of the strains in experimental infections (Weiss *et al.*, 1983) or in man.

Materials and methods

Bacterial strains

The following strains were used: *B. pertussis* phase I (5 strains), 18-323 (NCTC 10739), L84I (NCTC 11089), 18334, 165, Tohama; *B. pertussis* phase IV (2 strains), L84IV (NCTC 10901) and 11615; *B. parapertussis* (2 strains), NCTC 10520 and NCTC 5952; *B. bronchiseptica* (2 strains), 214 and 5376. Transposon-insertion mutants of *B. pertussis* (Weiss *et al.*, 1983) were provided by Dr A. Weiss, Microbiology and Immunology Department, Medical College of Virginia, Richmond VA, USA. These were: BP348 (Adc⁻, Hly⁻, adenylate cyclase- and haemolysin-deficient); BP349 (Hly⁻); BP353 (Fha⁻, deficient in filamentous haemagglutinin); BP357 (Ptx⁻, deficient in pertussis toxin); BP347 (Vir⁻, deficient in adenylate cyclase, haemolysin, pertussis toxin, filamentous haemagglutinin and heat-labile toxin).

B. avium strains P-4084, P-4091, P-4148, P-4480 (Rimler and Simmons, 1983) were provided by Dr R.B. Rimler, National Animal Diseases Center, Ames, Iowa, USA.

Media

Bordet-Gengou (BG) plates were used for routine growth of *Bordetella* spp. and for assessment of haemolytic activity. The plates consisted of a thick layer of BG Agar Base (Gibco) with a thin overlay of BG agar base containing defibrinated horse blood 20% v/v.

Cyclodextrin solid medium (CSM) was a modification of the medium of Imaizumi *et al.* (1983) and contained (g/L) monosodium glutamate 10.7 g, L-proline 0.24 g, NaCl 2.5 g, KH₂PO₄ 0.5 g, KCl 0.2 g, MgCl₂·6H₂O 0.1 g, CaCl₂ 0.02 g, Tris 6.1 g, casein hydrolysate peptone 5 (Gibco) 0.5 g, 2,6-0-dimethyl β -cyclodextrin (Me β CD) 1.0 g and Bacto-Agar (Difco) 18 g, in distilled water. It was adjusted to pH 7.4 then autoclaved at 121°C for 15 min. A supplement containing L-cysteine 0.04 g, FeSO₄·7H₂O 0.1 g, ascorbic acid 0.02 g, nicotinic acid 0.004 g, and reduced glutathione 0.15 g in 10 ml of distilled water was sterilised by membrane filtration and added to each litre of basal medium. This modified medium contained ten times more FeSO₄ than in the original formulation which, in our hands, supported the growth of <1% of the colonies detected on BG medium. With the increased FeSO₄, the plating efficiency was similar to that on BG. Me β CD was kindly provided by Dr J. Shimizu, Teijin Ltd, Chiyoda-ku, Tokyo, Japan.

A high nicotinic acid medium was used for growth of phenotypically altered C-mode cells (Pusztai and Joó, 1967; Wardlaw *et al.*, 1976; Schneider and Parker, 1982). Nicotinic acid was dissolved in a small amount of 1N NaOH, made up to 10 mg/ml with distilled water, sterilised by membrane filtration, and 5 ml was added to each 100 ml of CSM.

The differential media were prepared by adding Congo red, Trypan blue or haemin to CSM or to both layers of the BG medium. Haemin was dissolved in 1N NaOH and

stock solutions (1% w/v) of all three reagents were sterilised by autoclaving and added to the molten agar in the required amounts.

Plates were incubated in a moist atmosphere at 37°C for up to 7 days.

Characterisation of strains

Haemolysin production was assessed after growth on BG plates.

Haemagglutinin(s). Growth from the solid media was suspended in phosphate buffered saline (PBS), pH 7.2, to a concentration of 10 opacity units (OU) by comparison with the WHO 5th International Reference Preparation of Opacity (Perkins *et al.*, 1973); 50- μ l amounts were added to equal volumes of thrice-washed horse red blood cells (2% v/v in PBS) in U-shaped wells of plastic microtitration trays. After mixing, the trays were left at room temperature for 2 h before haemagglutination was assessed. Although *B. pertussis* contains at least two haemagglutinins, pertussis toxin and filamentous haemagglutinin (FHA), this test appears to measure only FHA since the mutant strain BP353 (Fha⁻, Ptx⁺) gave a negative result.

Histamine-sensitising factor (pertussis toxin). The bacterial suspensions (10 OU) prepared above were heated at 56°C for 30 min to destroy heat-labile toxin, and 0.5-ml amounts were injected intraperitoneally (i.p.) into groups of five adult HaM/ICR mice. After 5 days the animals were challenged i.p. with 0.5 ml of saline containing 3 mg of histamine dihydrochloride and survivors were counted after 3 h. This challenge dose of bacteria gave a clear distinction between phase-I *B. pertussis* (no survivors) and phase-IV and C-mode cells (all survived).

Heat-labile toxin activity was determined by skin reactions after subcutaneous injection of 3-4-week-old mice (Parton, 1986). Mice received injections of 0.05-ml amounts of the bacterial suspensions diluted to 2 OU in PBS. A reaction of >5 mm in diameter was taken as a positive response.

Virulence for mice by the intranasal route of infection was determined by the method of Pittman *et al.* (1980) with 3-week-old HaM/ICR mice.

X-bands. The presence of two major phase I (X-mode)-specific envelope proteins (X-bands) of mol. wt. c. 30 000 was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-cell suspensions (Brownlie *et al.*, 1985). A comparison of the protein profiles also served to confirm the identity of the isolates as *Bordetella* strains.

Growth on nutrient agar was assessed with thick plates of Nutrient Agar (Oxoid) incubated as described above.

Results

All of the phase-I *B. pertussis* strains gave red (CR⁺) colonies when stock cultures were streaked on to modified CSM containing Congo red 0.002% w/v (CSM-CR), whereas two phase-IV

strains gave pale (CR⁻) colonies. The transposon-insertion mutants of Weiss *et al.* (1983), BP348 (Adc⁻, Hly⁻), BP349 (Hly⁻), BP353 (Fha⁻) and BP357 (Ptx⁻), all produced CR⁺ colonies. However, BP347 (Vir⁻), deficient in a number of phase I-related properties, had the CR⁻ phenotype. The two *B. paraptussis* and two *B. bronchiseptica* strains tested were CR⁺ whereas all four *B. avium* strains were CR⁻.

With some of the CR⁺ strains, pale colonies were sometimes seen amongst the predominantly red colonies. These CR⁻ variants were subcultured on the same medium and tested for characteristics normally associated with phase-I *B. pertussis* strains and absent in BP347 and phase-IV strains (table). One CR⁻ variant from each of strains L84I, 165, 18334 and BP348 was fully characterised and they all had an identical phenotype. They lacked haemagglutinating activity, histamine-sensitising factor (pertussis toxin), heat-labile toxin and two major envelope proteins (X-bands) associated with phase-I (or X-mode) strains. They were non-haemolytic on BG medium and did not readily grow to single colonies on nutrient agar. Thus, they resembled BP347 and differed from phase-IV strains only in the last-mentioned characteristic. However, with the CR⁻ isolates and BP347, it was possible to obtain the growth of a few, isolated

colonies by streaking a heavy inoculum on to nutrient agar. This was never seen with phase-I strains. Several other CR⁻ clones were isolated from these same *B. pertussis* strains and all lacked haemolysin and haemagglutinin but these strains have not been characterised further.

The lack of phase I-related factors in the CR⁻ isolates was paralleled by loss of virulence for mice by the intranasal route. The CR⁻ variants of *B. pertussis* L84I and 165 caused no deaths in 3-week-old mice even at the highest dose tested (LD50 > 10⁸ cfu/mouse) whereas the LD50 values for the parent CR⁺ strains were <10⁷ cfu/mouse.

When phase-I (X-mode) *B. pertussis* strains L84I, 165 and 18334 were grown on CSM-CR containing a high concentration of nicotinic acid (500 µg/ml) to induce phenotypically altered C-mode cells, all of the resulting colonies were CR⁻. The C-mode state of these cultures was confirmed by the absence of haemagglutinating activity, heat-labile toxicity and X-bands in cell suspensions (table).

A spontaneous CR⁻ variant was isolated from each of the *B. paraptussis* and *B. bronchiseptica* strains and, where tested, the variants were shown to lack certain characteristics of the parents, namely haemolysin production, haemagglutinating activity and heat-labile toxin (table). As with *B. pertussis* strains, when grown on the high nicotinic acid

Table. Congo red phenotype and other characteristics of various *Bordetella* strains

Species and strains (number of strains tested)	Congo red bind- ing	Production of				Presence of X-bands in SDS-PAGE	Growth on nutrient agar	Mouse- virulence* (LD50)
		Haemolysin	Haemagglu- tinin(s)	Histamine- sensitising factor	Heat- labile toxin			
<i>B. pertussis</i>								
phase I (5)	CR ⁺	+	+	+	+	+	-	<10 ⁷ cfu/mouse
phase IV (2)	CR ⁻	-	-	-	-	-	+	...
BP347 (Vir ⁻)	CR ⁻	-	-	-	-	-	-	...
spontaneous CR ⁻ variant (4)	CR ⁻	-	-	-	-	-	-	>10 ⁸ cfu/mouse
phase I grown on high nicotinic acid medium (5)	CR ⁻	...	-	...	-	-	...	...
<i>B. paraptussis</i>								
parent (2)	CR ⁺	+	+	...	+	...	+	...
spontaneous CR ⁻ variant (2)	CR ⁻	-	-	...	-	...	+	...
<i>B. bronchiseptica</i>								
parent (2)	CR ⁺	+	+	...	...	...	+	...
spontaneous CR ⁻ variant (2)	CR ⁻	-	-	...	...	...	+	...

..., not tested or not applicable.

* Only two phase I strains, L84I and 165, and their CR⁻ variants were tested.

medium, the CR⁺ strains produced CR⁻ colonies which were non-haemagglutinating.

CR⁺ and CR⁻ colonies could also be differentiated by replacing the Congo red in CSM with Trypan blue 0.002% w/v, in which case blue or pale colonies respectively were produced, or with haemin (0.005% w/v) when dark brown or pale colonies were produced. However, the distinction between the two types was not as marked with haemin as with the dyes. It was also possible to use BG medium, instead of CSM, to observe dye binding. This was achieved by incorporating a higher concentration of the dye (0.01% w/v) in the growth medium, applying membrane filters (Whatman, cellulose nitrate, 0.45 µm) to the surface of the medium and streaking out across the membrane. After incubation, the red, blue or pale colonies were readily distinguished against the light background of the filter. Some component of BG medium appeared to interfere with haemin binding since even with the addition of haemin 0.01% w/v to the agar, CR⁺ strains grew as pale colonies on the membrane. It should be noted that with prolonged storage at room temperature or in the refrigerator, the CR⁻ strains began to take up the dyes and the difference between CR⁺ and CR⁻ became less apparent.

Discussion

B. pertussis resembles certain other bacterial species in that parent and variant strains can be differentiated by their ability to take up Congo red, Trypan blue or haemin from the growth medium. Phase-I *B. pertussis* strains had this ability. The phase-IV strains (Leslie and Gardner, 1931; Robinson *et al.*, 1986), the so-called avirulent-phase (Vir⁻) mutant BP347 (Weiss *et al.*, 1984), and the phenotypically altered C-mode cells (Lacey, 1960) lacked the ability, and these strains are known to be less virulent for mice than their parents. Moreover, spontaneous CR⁻ strains lacked the usual range of factors possessed by phase-I strains, namely production of haemolysin, heat-labile toxin, haemagglutinin(s), histamine-sensitising factor and two major envelope polypeptides, and two such strains were shown to be avirulent for mice.

The spontaneous CR⁻ variants of *B. pertussis* appear to be similar to the avirulent-phase (Vir⁻) variants described by Weiss and Falkow (1984) and to variants isolated by Peppler (1982) and Goldman *et al.* (1984) and are distinct from the classical phase-IV strains which grow readily on nutrient agar (Robinson *et al.*, 1986). They are presumed to have accumulated within the stock cultures of the

parent strains before streaking onto CSM-CR. No reversion of these strains to the CR⁺ phenotype has been observed. The concomitant loss of several phase I-related factors along with the Congo red phenotype in these isolates, in strain BP347, in phase-IV strains and under C-mode conditions (Parton and Wardlaw, 1975; Wardlaw *et al.*, 1976), suggests that a common regulatory mechanism is involved. Weiss and Falkow (1984) have proposed that a trans-acting positive effector, the product of the *vir* gene, is responsible for the expression of many of the putative virulence factors of *B. pertussis*. Inactivation of this gene by mutation or, as in BP347, by a single transposon Tn5 insertion, or in C-mode cells by environmental signals such as nicotinic acid, prevents expression of the putative virulence-associated genes. Thus the CR⁺ phenotype appears to be yet another characteristic whose expression is controlled by the *vir* gene.

A similar concomitant loss of activities and Congo red binding was observed in variants of *B. paraptussis* and *B. bronchiseptica* and under C-mode conditions suggesting that these closely-related species also have a functional *vir* gene. The recently described *B. avium* (Kerstens *et al.*, 1984), the causative agent of turkey coryza, appears to be more distantly related and all four strains tested were CR⁻. Moreover, although the organism produces a heat-labile toxin similar in activity to that of the other *Bordetella* species, expression of the toxin was not affected by growth in the high nicotinic acid medium (unpublished observations). It may be that this species does not have the same regulatory mechanisms as the other *Bordetella* species.

The virulence of CR⁻ variants of *Shigella*, *V. cholerae*, *E. coli* and *N. meningitidis*, in a chick embryo model, was restored by supplementation with iron, suggesting that the variants were defective in their ability to acquire iron *in vivo* (Payne and Finkelstein, 1977). Pathogenic organisms growing in the iron-restricted environment *in vivo* have developed mechanisms for assimilating protein-bound iron or for acquiring it from cell-free haem (Griffiths, 1983). The correlation between Congo red binding and haemin binding has been noted previously (Surgalla and Beesley, 1969; Prpic *et al.*, 1985) and the binding sites may be identical (Kay *et al.*, 1985; Daskaleros and Payne, 1987). Haemin and the related compound protoporphyrin IX were strong competitive inhibitors of Congo red binding to CR⁺ cells of *A. salmonicida*, presumably due to their structural similarity to the dye (Kay *et al.*, 1985). One possible function suggested for the Congo red binding site is to use protoporphyrin IX

(haemin without the ferric ion ligand) to strip iron from host iron-binding proteins. It is interesting to note that *B. pertussis* also may obtain its iron directly from host iron-binding proteins (Redhead *et al.*, 1987). With CR⁺ strains of *S. flexneri*, haemin binding and haemin uptake were independent since both CR⁺ and CR⁻ strains could use haemin as the sole source of iron. However, in CR⁺ strains, pre-treatment with haemin enhanced invasiveness for HeLa cells and it was suggested that this binding ability was in some way involved in attachment to host cells (Daskaleros and Payne, 1987).

Congo red binds to *A. salmonicida* by two different mechanisms; one is specific and of high affinity and the other is non-specific and probably due to hydrophobic interactions (Kay *et al.*, 1985). The surface of phase-I (CR⁺) *B. pertussis* cells is known to be hydrophobic whereas that of C-mode and phase variant strains is hydrophilic (Robinson *et al.*, 1983; Fish *et al.*, 1987). It is possible that differences in Congo red binding in *B. pertussis* are simply a reflection of differences in cell-surface hydrophobicity. Fish *et al.* (1987) suggested that filamentous haemagglutinin (FHA) is the main hydrophobic constituent of the cell, but it cannot be the only Congo red binding site since strain BP353, the FHA-deficient mutant of Weiss *et al.* (1983), gave CR⁺ colonies, as did the other mutants deficient in only one or two of the phase I-related factors. Differences in Congo red binding due to the presence or absence of different surface components in variant strains could possibly be dem-

onstrated by the quantitative dye-binding assay of Payne and Finkelstein (1977).

Clearly, the relationship between Congo red binding, iron acquisition, attachment and mouse-virulence in *B. pertussis* requires further investigation. We are currently trying to isolate, by chemical or transposon mutagenesis, CR⁻ variants of *B. pertussis* that have the full complement of other phase-I-related factors, in order to assess the significance of Congo red binding for mouse-virulence. Transposon mutagenesis has already been used to show the importance of pertussis toxin and adenylate cyclase in the mouse model (Weiss *et al.*, 1984). The Congo red phenotype may also be useful in studying the frequency of variation from the "virulent phase" to the "avirulent phase" as defined by Weiss *et al.* (1983) and the reversibility of the process (Weiss and Falkow, 1984). It must be borne in mind, however, that virulence of *B. pertussis* strains for mice cannot be equated with their virulence for man (Carter and Preston, 1981). The pathogenesis of whooping cough is complex and the contribution of the various phase-I-related factors remains speculative (Wardlaw and Parton, 1988). The screening of compounds for their modulating effect on *B. pertussis*, and the occurrence of variant strains during pertussis infection (reviewed by Coote and Brownlie, 1988) are other possible areas of investigation.

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