Capsulation gene loss and 'rescue' mutations during the Cap⁺ to Cap⁻ transition in *Haemophilus influenzae* type b

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Genes for *Haemophilus influenzae* type b capsule expression are duplicated to form a potentially unstable structure, *cap*, of directly-repeated chromosomal regions of approximately 17 kb. Capsule-deficient mutants arise in a two-stage process, initiated by *rec*-dependent reduction of this region from two copies to one. This recombinational event is usually lethal, only about 1/200 surviving to form slow-growing colonies of organisms that continue to synthesize polysaccharide but are defective in its export. A variety of secondary 'rescue' mutations within *cap* can occur to reduce polysaccharide synthesis and restore normal organism appearance and colony morphology.

Introduction

Haemophilus influenzae causes bacterial meningitis and other life-threatening invasive infections in young children. More than 95% of systemic infections caused by *H. influenzae* are due to organisms possessing the type b capsule (Turk, 1982), a polymer of ribose and ribitol 5-phosphate (PRP), which is an important virulence determinant (Moxon *et al.*, 1984).

It has long been recognized that PRP synthesis and type b capsule expression is readily lost by type b strains (Fothergill & Chandler, 1936). This commonly occurs in two steps (Buckmire, 1976). The first event gives rise to the class I mutant, which appears at a frequency of 0.1–0.5% (Hoiseth *et al.*, 1985). It is easily distinguished from its capsulate parent as organisms are highly pleomorphic and frequently filamentous, forming small, rough, non-iridescent colonies (see Fig. 1). A second event gives rise to class II variants, in which PRP synthesis is virtually abolished (Buckmire, 1982). Colonies of class II variants are large and smooth but non-iridescent on inspection by obliquely transmitted light (Fig. 1) and contain cocco-bacillary organisms of uniform appearance.

A region of the *H. influenzae* type b genome, the *cap* locus, is critically involved in capsule expression. This locus contains two directly repeated 17 kb DNA seg-

ments separated by a unique region of approximately 1.2 kb – the bridge region (Hoiseth *et al.*, 1986; Kroll *et al.*, 1988). When *EcoRI* digests of total genomic DNA of either class I or class II variants are probed with pSKH2, containing DNA cloned from the *cap* locus (Hoiseth *et al.*, 1985), a 9 kb band seen in the capsulate (b⁺) parent strain is lost. Otherwise the probing patterns are identical. This change in probe hybridization has been shown to correspond to reduction of *cap* to a single copy – the residual *cap* locus – through recombination between the direct repeats (Hoiseth *et al.*, 1986). In this rearrangement, the 9 kb *EcoRI* fragment spanning the junction between the *cap* repeats is lost, and with it the 1.2 kb bridge region (Fig. 2). The phenotype of class I variants can be reproduced by site-directed mutagenesis in the bridge region. This disrupts the gene *bexA* which has been shown to play a critical role in polysaccharide export (Kroll *et al.*, 1988) and supports the proposition that the defect in the spontaneous class I variants is in polysaccharide export rather than synthesis (Catlin & Tartagni, 1969; Buckmire, 1982). It has been suggested that intracellular accumulation of polysaccharide is responsible for the 'unhealthy' phenotype (Kroll *et al.*, 1988).

Using direct light and immunofluorescence microscopy with FITC-conjugated anti-PRP antibody (Buckmire, 1982; Catlin & Tartagni, 1969), class I and class II variants are readily distinguished from one another by the observation that the former are pleomorphic, with irregular accretions of intracellular capsular material,

Abbreviations: b⁺, type b capsulate; (s)BHI, (supplemented) brain heart infusion; *cap*, capsulation locus; FITC, fluorescein isothiocyanate; PRP, poly-ribosyl ribitol phosphate.

while the latter are uniform cocco-bacilli making very little or no PRP. The identity of *cap* probing pattern between class I and class II variants suggests that no large-scale rearrangement at the *cap* locus accompanies the change in phenotype. The change could come about through the down-regulation of PRP synthesis, with the implication of reversibility, or through mutation, either in biosynthetic genes within *cap* or in some other chromosomal locus altogether. In eight independent class II variants of the prototypic type b strain Eagan, we have found that the transition from class I to class II phenotype was the result of mutation in *cap* genes.

Methods

Bacterial strains. *H. influenzae* RM153 (Eagan) is a prototypic virulent type b strain (Anderson *et al.*, 1972). Eagan and its variants described in this work were grown in brain-heart infusion broth supplemented with 2 µg NAD ml⁻¹ and 10 µg haemin ml⁻¹ (sBHI). BHI plates were prepared with 1% (w/v) agar supplemented with 10% (v/v) Levinthal base (Alexander & Leidy, 1965) and strains were stored at -70 °C in sBHI broth and 20% (v/v) glycerol.

Phenotypic characterization of *H. influenzae*. Diluted cultures of *H. influenzae* were plated on to translucent sBHI agar and colony phenotype was assessed by viewing with obliquely-transmitted light as previously described (Hoiseth *et al.*, 1985). Immunofluorescent microscopy using rabbit anti-PRP antibody conjugated to FITC (Catlin & Tartagni, 1969) was used to assess the surface distribution of capsular antigen. Electron microscopy was performed as described previously (Kroll *et al.*, 1988).

Transformation of *H. influenzae*. *H. influenzae* was made competent for the uptake of linearized DNA by growth in sBHI broth followed by incubation in MIV medium (Herriott *et al.*, 1970) as described previously (Moxon *et al.*, 1984).

Preparation and analysis of *H. influenzae* chromosomal DNA. Total cellular DNA was prepared from 3 ml broth cultures as previously described (Moxon *et al.*, 1984). Genetic analysis of variants and transformants was accomplished by digestion of total cellular DNA with *Eco*RI, electrophoresis in agarose/Tris acetate gels and Southern blotting. The probe pUO38, containing a 18.2 kb *Bam*HI fragment from *cap* (Kroll *et al.*, 1988; and Fig. 2) was used to construct restriction maps of the residual *cap* locus in each variant.

Transforming DNA. Plasmids used for transformation contain *Eco*RI fragments from the *cap* locus cloned into the *Eco*RI site of pBR328, the name corresponding to the size of each fragment, and the plasmids pUO38 and pUO12 (Fig. 2).

Results

Independent class II variants can be transformed to the class I and capsulate phenotypes with cap DNA

A single colony of strain Eagan was grown to mid-exponential phase in sBHI and plated on to sBHI agar at a density of approximately 1000 colonies per plate. Eight class I colonies were selected by their characteristic appearance and separately streaked on to sBHI agar to

produce spontaneous class II variants (Fig. 1), and then one class II variant per plate was picked. This ensured that all the class II variants that formed the basis of this study arose through genetically independent events. The genotype of each class I and class II variant was checked by Southern blotting (Fig. 2). All were identical, with a residual *cap* locus containing only a single copy of the ~17 kb segment duplicated in the capsulate parent (data not shown). The class II variants were made competent for uptake of linearized DNA and exposed to the 18.2 kb *Bam*HI *cap* fragment excised from pUO38, seeking the class I phenotype in the products of transformation. In each case not only class I variants but also capsulate organisms were recovered. Transformants appeared identical to wild-type class I variants and capsulate organisms by several criteria: (i) colonial appearance; (ii) microscopic appearance after Gram and immunofluorescence staining (Fig. 1) and (iii) Southern blotting using pUO38 as a probe. Class I transformants had a probing pattern identical to the class II strains from which they were derived (Fig. 2). In contrast, capsulate transformants were found to have regained the 9 kb *Eco*RI fragment containing the bridge region, suggesting that the duplicated *cap* locus had been regenerated. The possibility of an appreciable rate of reversion of class II to class I phenotypes was refuted by examination of cultures of class II variants plated at low density on to sBHI agar. Approximately 20000 colonies were screened but none with the class I or capsulate phenotype was seen. The process of induction of competence for DNA uptake similarly does not induce such a phenotypic change.

Class II mutations are not confined to a single 'hotspot' in cap

To determine whether spontaneous mutations leading to the class II phenotype were localized to a 'hotspot' in *cap*, an attempt was made to transform the eight independent class II strains as before, but now using smaller non-overlapping fragments of *cap* DNA. In all cases the efficiency of transformation was found to be substantially reduced. Each strain could only be transformed by, at most, one of these fragments, and different fragments were necessary in each case (Table 1). Thus the secondary mutations leading to the class II phenotype do not appear to be limited to a single 'hotspot' but can occur at different locations throughout *cap*.

Recombinational reduction of cap to the class I genotype is often lethal

Recombinational events between adjacent identical ~17 kb segments of DNA would be expected to occur at

Fig. 2. (a) Physical map of the *cap* locus of the type b strain RM153. *Eco*RI sites are represented by the unlabelled vertical lines above the chromosome. Other restriction sites: X, *Xho*I; S, *Sal*I; B, *Bam*HI. Hatched bars represent the *cap* repeats. Black bars show the DNA contained in the plasmids pUO38 and pUO12. Recombinational reduction of the locus is also indicated. (b) Southern blot of *Eco*RI fragments of chromosomal DNA of b⁺ (track 1), class I (tracks 2, 4) and class II (tracks 3, 5) strains probed with nick-translated pUO38 showing the loss of the 9 kb *Eco*RI fragment in the class I mutant and no difference in the probing patterns of class I and class II mutants (only fragments over 4 kb are shown).

Fig. 3. Transmission electron micrograph through a randomly selected colony of RM153 showing the presence of spontaneous class I mutants characterized by terminal vacuolation (arrows). Bar, 0.5 μ m.

Table 1. *Effect of using different fragments of cap DNA to transform independently isolated class II variants*

(+) indicates that transformants were obtained; (–) indicates that no transformants were observed; ND, not done.

Strain	Transforming DNA:					
	pUO38	pUO12	9 kb	4.4 kb	2.7 kb	2.1 kb
5566	+	ND	+	–	–	–
5567	+	+	–	–	+	–
5569	+	ND	–	+	–	–
5570	+	–	–	–	–	–
5571	+	–	–	–	–	–
5574	+	–	–	–	–	–
5576	+	+	–	–	–	–
5577	+	–	–	–	–	–

a substantial frequency, and the occurrence of spontaneous class I variant colonies at a rate of only about 0.5% from cultures of type b strains suggested that organisms with the residual *cap* genotype might be of reduced viability. This is consistent with the observation of slow growth and 'sick' appearance of organisms making up class I colonies, choked with intracellular capsular material (Kroll *et al.*, 1988). This was tested by direct electron microscopic examination of dispersed spreads of organisms obtained from exponential cultures of type b strains, seeking the vacuolated export-defective class I variant phenotype at the organism level (Kroll *et al.*, 1988) (Fig. 3). In blind screening, 91 of 450 Eagan organisms were vacuolated (20%), in contrast to spreads of class II variants, in which no vacuolation was seen.

Discussion

We have shown that the reduction of the type b *cap* locus direct repeats, with loss of the bridge region, is often lethal. The organisms that do survive produce small, slow-growing colonies. This is associated with accumulation of capsular polysaccharide within the organism and that has been speculated to cause the abnormal phenotype through interference with normal cell growth and division (Kroll *et al.*, 1988). Further mutations within the *cap* locus of class I bacteria give rise to organisms of the healthy, unencapsulated class II phenotype. These organisms no longer accumulate intracellular polysaccharide, suggesting that mutations have impaired its synthesis. All the examples of these mutations studied here could be corrected by transformation with cloned *cap* DNA from a b⁺ strain to produce class I organisms again. This strongly suggests that the class I to class II transition is not regulated by genes outside the *cap* locus. Independently derived class II strains have mutations in different parts of their residual *cap* locus since examples can be transformed to class I by different cloned segments. There appears, therefore, to be no single 'hotspot' for secondary mutations within *cap*.

The electron microscopic quantification suggests that in an exponential culture of a type b strain, about 20% of organisms have the class I phenotype. However, only about 0.5% are detectable at the colony level; 99.5% of class I organisms die. The mutations leading to the class II colonial phenotype can thus be seen as 'rescue' mutations. The cessation of synthesis of capsular

material by any of a number of mutations within *cap* can apparently restore normal cellular appearance and growth rate.

The duplication of *cap* genes generally found in type b strains is not necessary for capsule production (Kroll & Moxon, 1988), and strains with a deleted region in one repeat are still b^+ (Ely *et al.*, 1986; Kroll & Moxon, 1988). We can now predict that such strains will show an altered pattern of capsule loss compared to the wild-type, and that different patterns will result from deletion of a *cap* DNA segment depending on whether it is located in the left or right repeat. The possibilities are illustrated in Fig. 4. In example 1, a deletion is located in the left repeat, x kb from the left end. Recombinational reduction of *cap* between homologous regions in the left x kb will result in a regular class I phenotype, but in the right y kb will result in a residual locus which retains the deletion and is therefore at a stroke defective in both polysaccharide synthesis and export and thus has the class II phenotype. If, as shown, $x \ll y$, and the simplifying observation is made that there are no 'hotspots' for recombination, the phenomenon observed macroscopically should be an unstable capsulate phenotype, readily giving rise to class II mutant colonies and only very rarely class I, as has indeed been found (Kroll & Moxon, 1989). As a numerical example, with $x = 3$ kb, the deletion = 1 kb and $y = 13$ kb, class II colonies are predicted at 20% of $13/16 = 16.3\%$, while class I colonies are predicted at only 0.5% of $3/16 = 0.1\%$. If the same deletion is located in the other repeat (Fig. 4, example 2) a very different situation is predicted. Now the genotype retaining the deletion is rare. With $x \ll y$, the rate of production of class I colonies should be near normal. However, even a low rate of production of the class II genotype will translate into the same rate of class II colonial phenotype if there is no impediment to proliferation, so we can predict an unstable capsulate phenotype with both class I and class II mutant colonies, the latter albeit at a lower rate than in example 1. Taking the same values for x and y , now class I colonies are predicted at 0.5% of $13/16 = 0.4\%$, but class II at 20% of $13/16 = 3.8\%$. Such differences were observed by Ely *et al.* (1986), who speculated that the different apparent stabilities of the capsulate phenotype between members of such pairs of mutants might reflect functional differences between the two repeats in *cap*. While this is certainly possible, we now offer this alternative simpler explanation based on our better understanding of class I and II mutants.

In conclusion, the unexpectedly low rate with which Rec-mediated capsule loss appears to occur in *H. influenzae* type b can be explained by the greatly reduced viability of class I variants. Healthy capsule-deficient progeny only arise after a second 'rescue' mutation. The relative importance of class I and class II variants in the

Fig. 4. Possible outcomes of recombinational reduction of *cap* in strains with asymmetric *cap* deletions. See text for details.

Haemophilus population is unknown. Class II variants are occasionally found among the non-typable *Haemophilus* that colonize the human respiratory tract. Such non-capsulate organisms appear to adhere better to human epithelial cells than their capsulate parents (St Geme & Falkow, 1991). Thus, it may be that the 'bottleneck' that the class I phenotype imposes on the flow of organisms from capsulate to non-capsulate confers an advantage by ensuring that the population of capsulate organisms is not overwhelmed by capsule-deficient mutants, which cannot be restored to the capsulate phenotype except by transformation.

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