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Capsulation and Gene Copy Number at the *cap* Locus of *Haemophilus influenzae* Type b

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Although more than 98% of natural isolates of *Haemophilus influenzae* type b carry a duplication of 17 kilobases (kb) of DNA at the chromosomal capsulation locus, only one copy is required for capsulation. In one laboratory-derived and two clinical type b strains, the capsulation locus had a single copy of this 17-kb segment, together with 1.3 kb of DNA identified as lying between the repeats of the duplicated locus. This 1.3 kb appears to be crucial for capsule production, since strains lacking it, although retaining a 17-kb segment, were capsule deficient. On comparing capsule polysaccharide production by these three type b strains with that by a prototypic type b strain with a duplicated locus, a gene dosage effect was demonstrated, with a halving of detectable polysaccharide in the single-copy strains. Despite this reduction in polysaccharide, these strains retained virulence potential as evidenced by bacteremia and meningitis in infant rats. As well as subserving augmented capsule polysaccharide production, a duplicated configuration of the type b *cap* locus endows strains with genetic instability not found in capsulate single-copy variants. We speculate that a survival advantage might be conferred on strains carrying a duplication at this locus as a result of gene dosage, the genetic instability of the locus, or both.

Haemophilus influenzae causes bacterial meningitis and other life-threatening infections in young children and represents a significant health hazard all over the world. Type b strains are responsible for over 95% of invasive infections caused by *H. influenzae* (25), and the type b capsule, a polymer of ribose and ribitol-5-phosphate (PRP) (8), has been shown to be an important determinant of virulence (14, 15).

A region of the *H. influenzae* type b chromosome, termed the *cap* b locus, that is involved in the expression of capsule has been shown to contain a duplication of an approximately 17-kilobase (kb) DNA segment, organized in a directly repeated configuration (12). Loci within each copy are involved in capsule production (9). In an analysis of 163 clinical isolates of *H. influenzae* type b from all over the world, this duplicated configuration of *cap* b was found in almost all strains (2), and it will be referred to here as the wild-type capsulation locus. However, two clinical isolates of type b strains have been identified which showed variant patterns of Southern hybridization with a *cap* b probe. One of these, RM1071, showed a hybridization pattern of *Eco*RI restriction fragments superficially very similar to that exhibited by spontaneous capsule-deficient mutants of serotype b strains, in which the *cap* b locus is known to be reduced to a single copy (11, 12). The other clinical isolate, RM8055, showed a hybridization pattern of *Eco*RI restriction fragments indistinguishable from that exhibited by a third strain, RM135, which is a type b laboratory transformant of strain Rd (a capsule-deficient mutant of a serotype d strain) (26).

We report here the results of an analysis of the *cap* loci, PRP production, and virulence potential of these three type b strains and their comparison with a prototypic type b strain (Eagan).

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MATERIALS AND METHODS

Media and culture conditions. *H. influenzae* strains were grown in brain heart infusion broth supplemented with 2 μg of NAD and 10 μg of hemin per ml. Translucent brain heart infusion plates were prepared with 1% agar and supplemented with 10% Levinthal base (1). Colony phenotypes were assessed by viewing with obliquely transmitted light as previously described (11).

Bacterial strains. The strains used are listed in Table 1. Strains were stored long term at −70°C in supplemented brain heart infusion broth containing 20% glycerol.

Transformation. Recipient strains were made competent by growth in supplemented heart infusion broth followed by incubation in the MIV medium of Herriott et al. (10) as previously described (14). Chromosomal DNA from RM2063 was used to transform strains to the streptomycin resistance phenotype. Transformants were detected by growth on supplemented brain heart infusion plates containing 200 μg of streptomycin per ml.

Preparation and analysis of genomic DNA. Total cellular DNA was prepared from 3-ml broth cultures as previously described (11, 14). Mapping of the *cap* locus was accomplished by restriction endonuclease digestion of total cellular DNA, electrophoretic separation of DNA fragments on 0.7% agarose-Tris acetate gels, transfer of the DNA to nitrocellulose filters (23), and hybridization to the nick-translated (20) [α-^{32}P]dCTP-labeled DNA probe pUO38. pUO38 contains DNA from the *cap* region of a wild-type strain of *H. influenzae* type b (see Fig. 2), subcloned in this laboratory from the cosmid clone pSKH3 (12) kindly provided by S. K. Hoiseth.

Quantitation of capsule polysaccharide (PRP). To compare PRP produced by different bacterial strains, it was necessary to develop methods to assay extracellular and cell-associated PRP at a defined stage of bacterial growth. The mid-exponential phase (organism density of 2×10^8 to 9×10^8

TABLE 1. *H. influenzae* strains used

Strain	Description
RM154	Spontaneous streptomycin-resistant mutant of Eagan, a prototypic type b strain (3)
RM804	Spontaneous capsule-deficient mutant of RM154 (this work)
RM1071	Clinical isolate (Alabama, 1968)
RM8055	Clinical isolate (Arizona, 1984)
RM135	Type b capsular transformant of Rd (same as Rd^-/b^+:O2 of Zwahlen et al. [26])
RM2063	Spontaneous streptomycin-resistant mutant of a type b clinical isolate in our collection

CFU/ml) was chosen in an attempt to ensure as uniform a population of bacterial cells as possible. To minimize contamination with nonviable (and so in these experiments uncountable) cells, cultures were set up by diluting 0.1 ml of an exponentially growing culture 100-fold with fresh medium. After regrowth to exponential phase, the viable count was measured and the cells were pelleted by centrifugation for 15 min at 12,000 × *g* at 4°C. In broth cultures of type b strains, soluble PRP is released into the culture medium during growth (4). A portion of supernatant was pipetted carefully from the top of the tube, so as to avoid contamination with cells, and stored for assay of the PRP content. Cellular surface-associated PRP was prepared from the cell pellet by incubation in EDTA by the method of Buckmire (6) as follows. The rest of the supernatant was removed and discarded, and the cells were washed with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 7.5). The washings were subsequently shown to contain very little PRP, as Buckmire also found. After being washed, the cells were resuspended in the same buffer, now also containing 10 mM EDTA, and incubated unshaken for 1 h at 37°C. Under these conditions, cellular surface-associated PRP is released into the buffer; it has been shown that very little residual PRP remains in the pellet of denuded cells left (6). After centrifugation as before, a portion of supernatant was removed for assay. Samples were stored briefly at 4°C or for longer periods at −20°C until assay. Under the conditions used, no detectable reduction in polysaccharide concentration with time was found.

The PRP concentration was measured by enzyme-linked immunosorbent assay (17) and was related for each sample to the original culture density. The capture antiserum coating the plates was burro anti-PRP (a gift of J. B. Robbins), and bound antigen was detected with horseradish peroxidase-conjugated rabbit anti-PRP (a gift of R. Yolken). *O*-Phenylenediamine was used as the peroxidase substrate at a final concentration of 0.1 mg/ml in citrate buffer. The light absorbance after the reaction was stopped with acid was measured at 490 nm.

A reference sample giving a strong positive reading in the assay was used to standardize plates, and the enzyme-linked immunosorbent assay was calibrated with a solution of purified PRP (a sample of *H. influenzae* type b vaccine kindly provided by Praxis Biologics [*b* CAPSA I, lot MO38AL; Praxis Biologics Inc., Rochester, N.Y.]).

Animal studies. Barrier-sustained litters of 5-day-old Sprague-Dawley rats were supplied by Harlan-OLAC (Oxford, United Kingdom). For assessment of bacterial virulence, litters were reduced to 12 pups and redistributed to the dams after being mixed, and washed suspensions of *H. influenzae* organisms were inoculated intraperitoneally into groups of animals (22). Bacteremia was assessed by counting CFU in 10-μl blood samples taken from the tail vein 48 h and 5 days after inoculation. Cerebrospinal fluid (CSF) was obtained by cisternal puncture at 5 days after inoculation, and bacteria were counted as CFU/10 μl. CSF samples were not visibly contaminated with blood, and meningitis was diagnosed by a bacterial count in CSF comparable to or greater than that in blood.

RESULTS

Physical mapping of the *cap* locus. Autoradiographs of Southern blots of *Eco*RI-digested chromosomal DNA from the type b strains RM1071, RM8055, and RM135, probed with pUO38, show obvious differences from that for the prototypic type b strain RM154, since they lack homology of the probe to 20- and 9-kb fragments (Fig. 1). In RM1071 a fragment a little larger than 20 kb was seen, while RM8055 and RM135 each had a 6.8-kb fragment hybridizing to probe. Otherwise the probing pattern was the same as for RM154. The hybridization pattern for RM804 is also shown. This spontaneous capsule-deficient laboratory mutant of RM154 (see below) also lacked a 9-kb fragment with probe homology, but the autoradiograph otherwise appears the same as that for RM154. Similar probing of these DNAs singly and doubly digested with the indicated restriction endonucleases allowed the construction of the physical maps shown in Fig. 2.

Identification of repeated *Pvu*II and *Nco*I sites delineated the extent of the repeats to at least 17 kb of apparent homology. The central *Sca*I site was situated in the residual 1.3 kb lying between the mapped extent of the repeats, and no analogous site was found at the right or left end of *cap* b. The sequence of restriction sites *Pvu*II-*Sca*I-*Nco*I character-

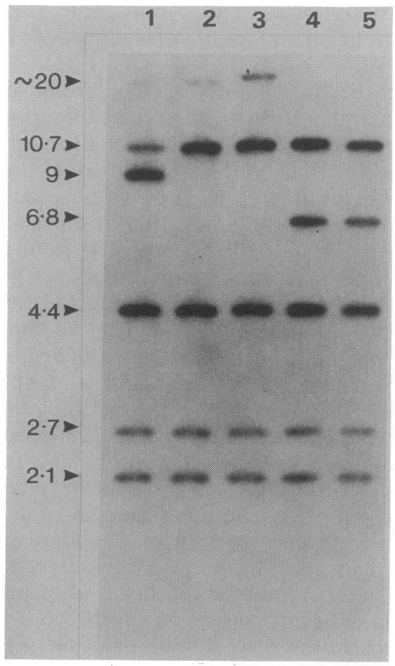

FIG. 1. Southern blot of *Eco*RI-digested chromosomal DNA from different strains of *H. influenzae* probed with [α-^{32}P]dCTP-labeled pUO38, a probe with homology to all *Eco*RI fragments in *cap* b. Lanes: 1, RM154 DNA; 2, RM804 DNA; 3, RM1071 DNA; 4, RM8055 DNA; 5, RM135 DNA. The sizes of fragments in the chromosome of RM154 with homology to probe are indicated on the left in kilobases.

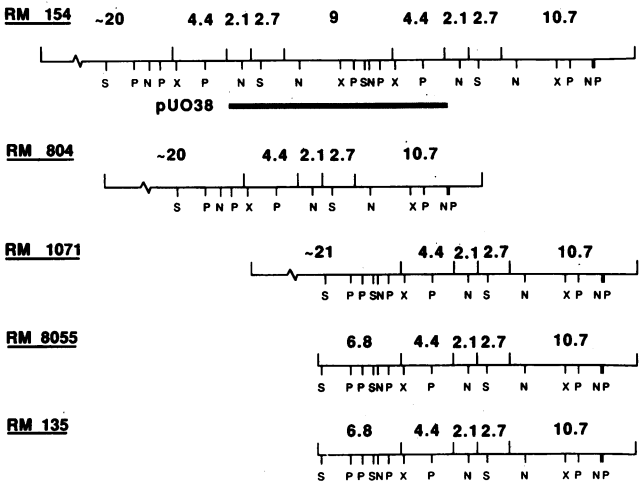

FIG. 2. Physical maps of the chromosomal capsulation loci of type b strains RM154, RM1071, RM8055, and RM135 and of RM804, a capsule-deficient derivative of RM154. *Eco*RI sites are represented by unlabeled vertical lines above the chromosome, and the sizes of *Eco*RI fragments are indicated in kilobases. Other restriction sites are indicated below the chromosome as follows: S, *Sca*I; P, *Pvu*II; N, *Nco*I, X, *Xho*I. The bridge region, with its identifying *Pvu*II-*Sca*I-*Nco*I sequence, is highlighted. DNA cloned from *cap* b in pUO38 is indicated below the *cap* locus of RM154.

ized this 1.3-kb element, which will be referred to here as the bridge region. It is shown highlighted in Fig. 2.

The residual *cap* locus in RM804 has only one copy of the 17-kb element, and its map does not contain the *Pvu*II-*Sca*I-*Nco*I sequence. A locus with this map has been demonstrated in a series of 22 independent capsule-deficient variants of type b strains from all over the world (unpublished results).

The physical maps of the *cap* loci of the three type b strains RM1071, RM8055, and RM135 are very similar to each other. In each, as in the noncapsulate strain RM804, there is one copy only of the element which is duplicated in the *cap* region of RM154. In contrast to the situation found for RM804, however, the bridge region is retained. Mapping with *Xho*I showed that in each case, by comparison with RM154, virtually all of the left repeat is missing, while previously recognized sites extending beyond it to the left are retained. These strains are referred to here as single-copy variants, and their *cap* genotype is called single copy.

Synthesis of type b capsule polysaccharide (PRP). RM154 incubated for 16 to 18 h on translucent plates gave rise to colonies that appeared brilliantly iridescent as a result of refraction of obliquely transmitted light by encapsulated organisms. In contrast, an additional 6 h of incubation was regularly required to bring colonies of the single-copy variants to a similar appearance. To explore the basis for this, mean generation times were determined for all strains. No difference was found between the wild type and the single-copy variants, all of which doubled in 27 to 28 min in exponentially growing broth cultures. The delay in development of colony iridescence in the single-copy variants was therefore unlikely to be attributable simply to a slower rate of growth. The production of capsule polysaccharide by each strain was therefore examined.

In exponential growth in liquid culture, RM154 released 3.7 ± 0.35 ng of PRP per 10^6 CFU (mean of five experiments ± standard error of the mean). Within the range of culture density of 2×10^8 to 9×10^8 CFU/ml, no systematic variation of PRP production with bacterial count was seen. The single-copy variants RM1071, RM8055, and RM135 released significantly less (about half as much) PRP ($P \leq 0.001$ by Student's *t* test) (Table 2). No PRP was detected from the capsule-deficient strain RM804. Treatment with EDTA caused a further release of PRP, between 2.6 and 4.4 times that found in the culture supernatant. Whereas EDTA-treated RM154 cells released 11.5 ± 1.2 ng of PRP per 10^6 CFU, RM1071, RM8055, and RM135 again released significantly less (about half as much) PRP ($P \leq 0.001$ by Student's *t* test). RM804 lacked detectable PRP.

Virulence studies. The virulence of the capsulate strains RM1071, RM8055, and RM135 was assessed in comparison with that of RM154 by their capacity to cause sustained bacteremia and meningitis after intraperitoneal inoculation of 5-day-old infant rats. The results of a representative experiment, in which groups of nine animals were inoculated with ca. 200 organisms of each strain, are shown in Fig. 3. At 48 h, approximately the same number of animals in each group was bacteremic. However, the animals infected with RM154 or RM135 had very heavy bacteremia (1,000 to 9,000 CFU/10 μl), whereas those given RM8055 or RM1071 showed markedly lower bacterial counts (1 to 66 CFU/10 μl).

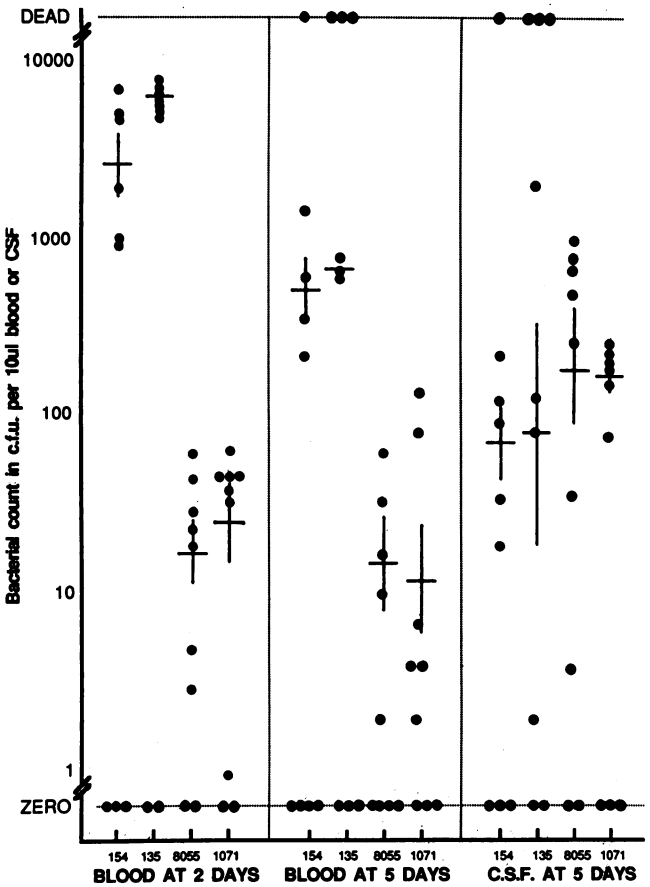

FIG. 3. Comparative virulence of type b strains RM154, RM135, RM1071, and RM8055 after intraperitoneal inoculation into 5-day-old infant rats. Nine animals were inoculated with each strain (200, 300, 100, and 200 CFU, respectively), blood was drawn at 2 and 5 days, and CSF was sampled at 5 days. Individual bacterial counts are displayed on a logarithmic scale, with the mean and standard error of the mean shown for positive cultures in survivors at each sampling.

TABLE 2. Capsule polysaccharide (PRP) in the supernatant and associated with bacterial cells in exponentially growing cultures of *H. influenzae*

Strain	PRP (ng/10^6 CFU)[a]	
	Culture supernatant	Cellular, EDTA releasable
RM154	3.7 ± 0.35 (5)	11.5 ± 1.2 (5)
RM135	1.3 ± 0.25 (6)	5.6 ± 0.7 (6)
RM1071	2.1 ± 0.15 (6)	5.4 ± 0.7 (6)
RM8055	1.5 ± 0.15 (5)	6.6 ± 0.15 (5)
RM804	ND	ND

[a] PRP in exponentially growing culture, as determined by enzyme-linked immunosorbent assay. Results are means ± standard errors of the mean; numbers in parentheses are the number of independent samples assayed. ND, None detected.

The geometric means for bacteremia with RM8055 (18 CFU/10 μl) and RM1071 (27 CFU/10 μl) differed significantly from that with RM154 (2,700 CFU/10 μl) ($P < 0.001$ by Student's t test).

Five days after inoculation, one and three animals that had received RM154 and RM135, respectively, had died, while there had been no deaths among animals infected with RM1071 or RM8055. Again, the levels of bacteremia in survivors were strikingly higher in recipients of RM154 and RM135 (geometric means of 530 and 750 CFU/10 μl, respectively) than in those given RM1071 and RM8055 (geometric means of 12 and 15 CFU/10 μl, respectively). However, every animal infected with RM8055 had meningitis (although two had no detectable bacteremia), while of those given RM1071, although one animal had apparently cleared the infection, the other six were bacteremic and had meningitis too. Bacterial counts in CSF were comparable for all four strains.

To investigate the possibility that such virulence in single-copy variant strains resided in a subpopulation of organisms with a wild-type *cap* locus, DNA was prepared from organisms that were cultured from the blood and CSF of animals infected with RM8055, RM1071, and RM135, and the *cap* locus was mapped. The single-copy *cap* genotype was retained in all of these isolates (data not presented).

Phenotypic variation: loss of capsule expression. Type b organisms with the wild-type *cap* locus readily become capsule deficient in vivo and in vitro (7, 11, 18) in a process dependent on an intact *rec* function and accompanied by reduction of the *cap* duplication to a single copy (12). RM1071, RM8055, and RM135, although lacking the large duplication at *cap*, presumably retain small direct repeats at each end of the locus, as evidenced by the duplication of the *Pvu*II sites corresponding in the wild type to those at the right-hand end of each repeat as drawn in Fig. 2. As such DNA might provide the basis for a similar *rec*-dependent event in the single-copy *cap* locus, the stability of capsule expression was assessed by colony screening.

Of 12,000 colonies of RM154 screened, 51 (0.4%) were noniridescent (i.e., contained capsule-deficient organisms), a frequency agreeing well with previously published data (11). In contrast, on screening 34,000 RM1071, 11,000 RM8055, and 42,000 RM135 colonies, no such variant colonies were found. In each case the difference is highly significant ($P \ll 0.001$) by Fisher's exact test).

The integrity of the *rec* system in the single-copy strains was established by transformation to streptomycin resistance with donor DNA from RM2063. In each case, transformants were readily isolated by selection on antibiotic-containing plates at frequencies several orders of magnitude above the spontaneous mutation rate (data not presented).

DISCUSSION

This work establishes that the direct-repeat configuration of the *cap* locus of *H. influenzae* type b, found in more than 98% of natural isolates (2), is not required for capsule production. Previous mapping of the wild-type locus (9, 12) had shown the duplication. In the present work, mapping with *Nco*I and *Pvu*II has allowed further delineation of the extent of the repeats to at least 17 kb of apparent homology. This reduces the maximum size of the bridge region between them, which contains DNA not assigned to either repeat, to 1.3 kb. As previously reported (9), a unique *Sca*I site lies between the repeats, so a *Pvu*II-*Sca*I-*Nco*I sequence (highlighted in Fig. 2) characterizes and serves to identify the bridge region.

In the present work we found that the clinical type b isolates RM1071 and RM8055 and the laboratory transformant RM135 differed from the wild type in having an unduplicated *cap* locus. At *cap* in these strains, only one 17-kb DNA segment is found, together with the 1.3-kb bridge region. In carrying the bridge region, the single-copy *cap* locus is also different from the residual *cap* locus in spontaneous capsule-deficient mutants of wild-type strains, which lack this element. These findings suggest that DNA in the bridge region may be critically important in capsule production, either by containing a gene(s) the products of which are necessary for capsule expression or by containing regulatory DNA sequences involved in the expression of genes within the 17-kb elements. Characterization of DNA in the bridge region is under way, and there is preliminary evidence for the presence of a coding sequence there, with a gene product involved in some way in the export of capsular polysaccharide (unpublished data).

The importance of DNA in the 17-kb segment for type b capsulation is confirmed pari passu. Symmetrical 1.2-kb deletions within the 4.4-kb *Eco*RI segments have been shown to abolish capsulation (9), and a point mutation within the span of this 1.2 kb has the same effect (9). This mutation is correctable by transformation with DNA having homology to the chromosome of type b or type a strains, but not the other serotypes (11). This suggests a likely role for the putative gene product encoded in this 1.2 kb in ribitol modification, this sugar being a component of types a and b but not of other capsular polysaccharides. DNA adjacent to this region appears to be involved in the synthesis of type b capsular polysaccharide alone (S. Ely, J. Tippett, and E. R. Moxon, unpublished results). Should these preliminary observations be confirmed, the overall organization of the capsulation locus will begin to resemble that found for *Escherichia coli* K1 (5), in which contiguous DNA segments encode, in turn, the general function of polysaccharide transport, the specific function of synthesis of a particular K polysaccharide, and a general postsynthetic modification function.

Our results show a gene dosage effect of the *cap* duplication. RM154 in the exponential phase of growth carried twice as much surface-associated PRP per organism and released twice as much PRP into the culture medium as any of the three single-copy variants. Either cell-associated or cell-free PRP might play a part in the pathogenetic sequence of the organism's interaction with humans. Cell-associated PRP, as capsule, has been shown to endow the organism with resistance to phagocytosis by polymorphonuclear leukocytes in

vitro in the absence of anticapsular antibody (16). Cell-free PRP might enhance virulence by saturating the capacity of opsonic host defenses, as has recently been suggested as a potential complication of immunization with this polysaccharide. Although the single-copy type b strains differed from the wild type in both these respects, we did not find a straightforward correlation between PRP production and the virulence of organisms in the infant rat bacteremia-meningitis model. RM135 appeared highly virulent, producing massive bacteremia on intraperitoneal inoculation that was indistinguishable from that with RM154. Studies with the same strain given intranasally have confirmed its virulence (26). In contrast, similar inocula of RM1071 and RM8055 gave rise to significantly lower bacteremia at 48 h. Nonetheless, at 5 days animals infected with these strains were still bacteremic and also had meningitis; within the CSF there was no apparent restriction on growth of any strain. The lack of difference between RM135 and the wild type in this model and the capacity of RM1071 and RM8055 to cause disease in a manner apparently rather different from that of the wild-type underscore the observation (13, 19, 24) that virulence depends on a number of factors, only one of which is capsule production.

If capsulation is possible without duplication in *cap*, how can the persistence of the wild-type locus be explained? Two direct consequences of the duplication have been established: augmented production of capsule polysaccharide and genetic instability.

It seems reasonable to ascribe biological advantage to augmented capsule polysaccharide production. We speculate that the capsule might have a role earlier in the host-parasite interaction than that of potentiating mucosal invasion (21). The gene dosage effect of the *cap* duplication could provide survival advantage to the organism in the external environment; since the spread of *H. influenzae* is by airborne respiratory droplets, having more of a hydrophilic capsule might be expected to reduce the chance of dehydration and enhance dissemination. Pilot studies in our laboratory have suggested that capsulate organisms remain viable for longer than noncapsulate organisms when air dried on various surfaces (unpublished results).

The capsulate phenotype appears to be relatively fixed in the single-copy variants RM1071, RM8055, and RM135. No capsule-deficient mutants were found after screening sufficient colonies to set the upper limit on this event at around 1 to 2% of that in the wild type. The rarity among naturally occurring strains of the genetically more stable single-copy *cap* locus and the worldwide predominance of the unstable, duplicated *cap* locus therefore appears paradoxical at first sight. However, the direct repeat can form the basis both for the reduction of *cap* and for the generation of triplications through unequal crossover between DNA strands during chromosomal replication. Provided that the basis for *cap* reorganization is through such inter- rather than intrachromosomal recombination, an advantage of gene dosage in *cap* b may remain uncompromised by its genetic instability. The direct repeat in *cap* might represent a metastable arrangement, regenerated via a triplication as rapidly as it is lost in the reduction to a single copy. Amplifications to triple and higher copy number have recently been found in *cap* b following transposon mutagenesis, and a strain derived in this way with a triplication in *cap* b produced more PRP than the parent from which it was derived. Futhermore, a clone of the *cap* locus from a cosmid bank of a type b strain has been shown to carry a triplication, suggesting that this may occur naturally (S. K. Hoiseth, personal communications). While capsule-deficient mutants are readily recognized by their colonial appearance and have a characteristic probing pattern with pUO38, mutants carrying triplications would not be detected by either approach. Quantitative assessment of PRP production should now allow detection of such organisms and further delineation of genetic variation at *cap* b.

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LITERATURE CITED

1. **Alexander, H. E.** 1965. The *Haemophilus* group, p. 724–741. *In* R. J. Dubos and J. G. Hirsch (ed.), Bacterial and mycotic infections of man. Pittman Medical Publishing Co. Ltd., London.
2. **Allan, I., M. R. Loeb, and E. R. Moxon.** 1987. Limited genetic diversity of *Haemophilus influenzae* (type b). Microb. Pathogen. **2**:139–145.
3. **Anderson, P., R. B. Johnston, Jr., and D. H. Smith.** 1972. Human serum activities against *Haemophilus influenzae* type b. J. Clin. Invest. **51**:31–38.
4. **Anderson, P., and D. H. Smith.** 1977. Isolation of the capsular polysaccharide from culture supernatant of *Haemophilus influenzae* type b. Infect. Immun. **15**:472–477.
5. **Boulnois, G. J., I. S. Roberts, R. Hodge, K. R. Hardy, K. B. Jann, and K. N. Timmis.** 1987. Analysis of the K1 capsule biosynthesis genes of *Escherichia coli*: definition of three functional regions for capsule production. Mol. Gen. Genet. **208**: 242–246.
6. **Buckmire, F. L. A.** 1982. Characterisation of the capsular antigen isolated from *Haemophilus influenzae* type b and two spontaneous non-encapsulated mutants, p. 171–183. *In* S. H. Sell and P. F. Wright (ed.), *Haemophilus influenzae*: epidemiology, immunology and prevention of disease. Elsevier Science Publishing Co., New York.
7. **Catlin, B. W.** 1970. *Haemophilus influenzae* in cultures of cerebrospinal fluid: non-encapsulated variants typable by immunofluorescence. Am. J. Dis. Child. **120**:203–210.
8. **Crisel, R. M., R. S. Baker, and D. E. Dorman.** 1975. Capsular polysaccharide of *Haemophilus influenzae* type b. J. Biol. Chem. **250**:4926–4930.
9. **Ely, S., J. Tippett, J. S. Kroll, and E. R. Moxon.** 1986. Mutations affecting expression and maintenance of genes encoding the serotype b capsule of *Haemophilus influenzae*. J. Bacteriol. **167**: 44–48.
10. **Herriott, R. M., E. M. Meyer, and M. Vogt.** 1970. Defined nongrowth media for stage II development of competence in *Haemophilus influenzae*. J. Bacteriol. **101**:517–524.
11. **Hoiseth, S. K., C. J. Connelly, and E. R. Moxon.** 1985. Genetics of spontaneous high-frequency loss of b capsule in *Haemophilus influenzae*. Infect. Immun. **49**:389–395.
12. **Hoiseth, S. K., E. R. Moxon, and R. P. Silver.** 1986. Genes involved in *Haemophilus influenzae* type b capsule expression are part of an 18-kilobase tandem duplication. Proc. Natl. Acad. Sci. USA **83**:1106–1110.
13. **Moxon, E. R.** 1985. Antigen expression influencing tissue invasion of *Haemophilus influenzae* type b. Bayer-Symp. **8**:17–29.
14. **Moxon, E. R., R. A. Deich, and C. Connelly.** 1984. Cloning of chromosomal DNA from *Haemophilus influenzae*. Its use for studying the expression of type b capsule and virulence. J. Clin. Invest. **73**:298–306.
15. **Moxon, E. R., and K. A. Vaughn.** 1981. The type b capsular polysaccharide as a virulence determinant of *Haemophilus influenzae*: studies using clinical isolates and laboratory transformants. J. Infect. Dis. **143**:517–524.
16. **Musher, D., A. Goree, T. Murphy, A. Chapman, J. Zahradnik, M. Apicella, and R. Baughn.** 1986. Immunity to *Haemophilus influenzae* type b infection in young adults: correlation of

bactericidal and opsonising activity of serum with antibody to polyribosylribitol phosphate and lipooligosaccharide before and after vaccination. J. Infect. Dis. **154**:935–943.

17. **Pepple, J., E. R. Moxon, and R. H. Yolken.** 1980. Indirect enzyme-linked immunosorbent assay for the quantitation of the type-specific antigen of *Haemophilus influenzae* b: a preliminary report. J. Pediatr. **97**:233–237.
18. **Pittman, M.** 1931. Variation and type specificity in the bacterial species *Haemophilus influenzae*. J. Exp. Med. **53**:471–492.
19. **Plaut, A. G.** 1983. The IgA_1 proteases of pathogenic bacteria. Annu. Rev. Microbiol. **37**:603–622.
20. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick-translation with DNA polymerase I. J. Mol. Biol. **113**: 237–251.
21. **Rubin, L., and E. R. Moxon.** 1983. Pathogenesis of bloodstream invasion with *Haemophilus influenzae* type b. Infect. Immun. **41**:280–284.
22. **Smith, A. L., D. H. Smith, D. R. Averill, Jr., J. Marino, and E. R. Moxon.** 1973. Production of *Haemophilus influenzae* b meningitis in infant rats by intraperitoneal inoculation. Infect. Immun. **8**:278–290.
23. **Smith, G. E., and M. D. Summers.** 1980. The biochemical transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl paper. Anal. Biochem. **109**:123–129.
24. **Sutton, A., R. Schneerson, S. Kendall-Morris, and J. B. Robbins.** 1982. Differential complement resistance mediates virulence of *Haemophilus influenzae* type b. Infect. Immun. **35**:95–104.
25. **Turk, D. C.** 1982. Clinical importance of *Haemophilus influenzae*, p. 3–9. *In* S. H. Sell and P. F. Wright (ed.), *Haemophilus influenzae*: epidemiology, immunology and prevention of disease. Elsevier Science Publishing Co., New York.
26. **Zwahlen, A., L. G. Rubin, and E. R. Moxon.** 1986. Contribution of lipopolysaccharide to pathogenicity of *Haemophilus influenzae*: comparative virulence of genetically-related strains in rats. Microb. Pathogen. **1**:465–473.