INFECTION AND IMMUNITY, June 1994, p. 2582–2589
0019-9567/94/$04.00+0

Vol. 62, No. 6

Phase Variation in Pneumococcal Opacity: Relationship between Colonial Morphology and Nasopharyngeal Colonization

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Received 22 December 1993/Returned for modification 10 March 1994/Accepted 30 March 1994

When colonies of encapsulated isolates of *Streptococcus pneumoniae* are viewed with oblique, transmitted light on a transparent surface, they are heterogeneous in appearance because of variation in opacity. There is spontaneous phase variation among at least three discernible phenotypes at frequencies from 10^{-3} to 10^{-6}. The ability to detect differences in opacity varies according to serotype, but variation is independent of capsule expression. Electron microscopy shows no difference in chain length but suggests that autolysis occurs earlier in the growth of the transparent variant. There was no identifiable difference in membrane protein profiles of opaque and transparent variants of the same strain. In an infant rat model of nasopharyngeal carriage, there was no significant colonization by opaque variants. Efficient and stable colonization by the transparent variants was observed, suggesting a selective advantage for this phenotype in the nasopharynx. In contrast, there was no difference in the incidence of bacteremia or in the 50% lethal dose among the variants following their intraperitoneal inoculation. These results suggest that phase variation which is marked by differences in colonial morphology may provide insight into the interaction of the pneumococcus with its host.

Streptococcus pneumoniae, or the pneumococcus, remains a leading cause of bacterial disease, particularly among the very young and very old. Recently, the clinical management of pneumococcal infection has become more difficult because of the appearance and widespread dissemination of strains resistant to penicillin and other antibiotics (11, 17). The spectrum of illnesses caused by the pneumococcus is a reflection of its ability to survive in various ecological niches in the human host. The organism resides in the nasopharynx, most commonly without adverse effect on its host, but may spread locally to cause upper or lower respiratory tract infection (4). In some circumstances, the organisms are able to enter the bloodstream from the nasopharynx via the cervical lymphatics, leading to bacteremia and, occasionally, infection of other organ systems.

The relationship of the pneumococcus to its host shares many features in common with encapsulated members of the genera *Haemophilus* and *Neisseria*, the major bacterial pathogens residing in the human respiratory tract which frequently cause bacteremia. For both the *Haemophilus* and *Neisseria* genera, a critical step in pathogenesis is the ability to adapt to the varied challenges encountered in the nasopharynx and blood by turning on and off the synthesis of key surface structures required in selective host environments (16, 35, 44, 45). In these pathogens, there is spontaneous, reversible expression or phase variation of structures which, in some cases, may be apparent as differences in colonial morphology. In fact, phase variation in colonial morphology has allowed the identification of several cell surface molecules of importance at certain key stages in the host-parasite interaction and thus has provided insight into adaptive strategies utilized by the bacterium in vivo (19, 36, 42, 44). In the case of the pneumococcus, differences in colonial morphology attracted the attention of early investigators largely because of the association between the smooth phenotype, encapsulation, and virulence, as well as observations about transformation first reported in 1928 by Griffith (3, 10, 14, 15, 28). Intrastrain variation in colonial morphology was also shown to be a factor in natural transformation by Avery, MacLeod, and McCarty (8).

Despite being the subject of many decades of investigation, little is understood about how the pneumococcus becomes established in the human nasopharynx and then makes the transition from its site of residence on the mucosal surface to the bloodstream. The only well-defined determinant of virulence for the pneumococcus, the capsular polysaccharide, of which there are 84 known types, is expressed constitutively and is required to evade phagocytosis once the organism has traversed the respiratory epithelium (7). Bacterial factors that contribute to colonization, the initial event in pneumococcal infection, are less well understood. Adherence to the mucosal epithelium has been proposed as a critical step in the maintenance of the pneumococcus in its ecological niche in the nasopharynx, and a glycoconjugate receptor on human pharyngeal epithelial cells has been partially characterized (1, 13). A recent report by Geelen et al. suggests a role for the peptidoglycan in adhesion to cultured endothelial cells (12). The role of pneumococcal cell wall components in adherence to epithelial surfaces has not been addressed.

Several studies have suggested that the expression of bacterial factors mediating colonization and adherence to epithelial cells varies at different stages during pneumococcal infection. A mouse model has been used to show that pneumococci cultured from different sites exhibit intrastrain differences in their ability to colonize the nasopharynx and cause infection when reintroduced into the host (43). Respiratory tract isolates have been shown to adhere better to human pharyngeal epithelial cells in vitro compared with those from blood or spinal fluid (2). However, phase variation of cell surface structures and its relationship to the pathogenesis of the pneumococcus, the subjects of this report, have not been described previously.

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TABLE 1. Strain characteristics and reversion frequency for pneumococcal opacity variants

Strain	Serotype or characteristics	Phenotype		Rate[a]	Source or reference
		Initial	Revertant		
P62	9V	Transparent	Opaque	2.5×10^{-3}	This study
		Opaque	Transparent	6.7×10^{-4}	
P6	9V	Transparent	Opaque	6.0×10^{-6}	This study
		Opaque	Transparent	1.7×10^{-5}	
P68	18C	Transparent	Opaque	1.0×10^{-4}	This study
		Opaque	Transparent	1.3×10^{-4}	
P24	+[b]	Transparent	Opaque	1.4×10^{-6}	This study
		Opaque	Transparent	6.8×10^{-6}	
D39S	2	Transparent	Opaque	$<5 \times 10^{-7}$	8
		Opaque	Transparent	$<5 \times 10^{-7}$	
RUP24	*lytA::ermC*	Opaque	Transparent	2.0×10^{-6}	41

[a] Expressed as the ratio of revertants divided by the number of nonrevertants. Values are the means of at least three separate determinations.
[b] +, unencapsulated mutant of a D39S strain.

MATERIALS AND METHODS

Bacterial strains, culture media, and chemicals. The strains examined in this study were isolates from blood cultures of patients with pneumococcal bacteremia sent to R. Austrian for serotyping and are listed in Table 1. Strain P24 is a phenotypic variant of strain R6, an unencapsulated laboratory-adapted mutant of an isolate of pneumococcus serotype 2 (34). Pneumococci were grown in Todd-Hewitt broth with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) or in a semisynthetic medium (C+Y medium) prepared as previously described (20). Broth cultures were streaked on tryptic soy broth solidified with 1% agar onto which 100 μl of catalase (6,300 U) (Worthington Biochemical Co., Freehold, N.J.) was added per plate. The strains employed in this study required an atmosphere of increased CO_2 for optimal growth on tryptic soy agar, which was provided in a candle-extinction jar (6). Colonial morphology was assessed with a stereo-zoom microscope with a 100-W halogen substage illuminator and adjustable-angle mirror after incubation of plates for 16 h at 37°C.

Rates of phase variation were determined by inoculation of a single colony into C+Y medium. Following growth to an optical density at 620 nm of 0.5, 10-fold serial dilutions of bacteria were plated on solid medium and the number of colonies of a phenotype different from that of the inoculum were enumerated. Generation time was determined by growth in semisynthetic and nutrient media at 37°C in an atmosphere of increased CO_2.

Chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise specified.

Electron microscopy. Overnight cultures of bacteria grown on tryptic soy agar were resuspended in phosphate-buffered saline (PBS [pH 7.2]) and washed in another volume of PBS. Plate-grown organisms were examined in order to compare differences in colony morphology with the appearance of cells in electron micrographs. The bacteria were fixed, sectioned, and further processed for electron microscopy according to previously described procedures (33). Thin sections of bacteria were examined in a JEOL 100 CX transmission electron microscope.

Qualitative and quantitative analysis of encapsulation. The capsular serotype of pneumococcal strains was determined by the Quellung reaction with specific antisera (Statens Serum-institut, Copenhagen, Denmark [27]). A modified enzyme-linked immunosorbent assay (ELISA) technique was used to assess the relative quantity of cell-associated capsular polysaccharide for each phenotype (26). PBS-washed organisms at mid-log phase were serially diluted twofold across a microtiter plate in PBS and incubated at 37°C for 4 h and then were incubated overnight at 4°C. Capsular polysaccharide antiserum diluted 1/5,000 was added to the washed plate, and the plate was incubated at 20°C for 4 h. After incubation of the washed plate with alkaline phosphatase-conjugated anti-rabbit immunoglobulin antibody (Bio-Rad Laboratories, Richmond, Calif.), the plate was developed in the presence of disodium *p*-nitrophenyl phosphate substrate (1 mg/ml) in diethanolamine buffer and the A_{405} was measured.

Analysis of membrane-associated proteins. Membrane fractions were prepared from sonicated bacteria as described elsewhere (29). Membrane-associated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5 to 15% gels and visualized by Coomassie blue staining. SDS-PAGE-separated membrane-associated proteins were also analyzed by Western blotting (immunoblotting) with polyclonal rabbit antisera raised to PBS-washed, formalin-treated opaque or transparent variants of a type 9V strain, P6.

Infant rat model of colonization and bacteremia. Synchronized pregnant Sprague-Dawley rats were purchased from Taconic Farms, Germantown, N.Y. Five-day-old infant rats were randomized among litters. For intranasal inoculations, 10 μl of PBS-washed mid-log-phase organisms adjusted to the desired density were inoculated into the left anterior naris. Colony counts were performed to ensure the inocula were of the desired density and phenotype. Animals receiving different phenotypic variants were housed in separate cages. The nasopharynx was cultured for viable pneumococci by the slow instillation of 20 to 40 μl of sterile PBS into the left naris and withdrawal of the initial 10-μl discharge from the right naris. This procedure ensured that the fluid had passed through the nasopharynx. The quantity and phenotype of organisms were determined by spreading the 10-μl nasal wash on tryptic soy agar containing catalase and neomycin (20 μg/ml) to minimize the growth of contaminating bacteria. To detect and quantify bacteremia, blood was cultured from a 10-μl sample obtained from the ventral tail vein.

To induce bacteremia, intraperitoneal inoculation was carried out with 100 μl of PBS-washed mid-log-phase bacteria adjusted to the desired density. Six pups each received from 10^1 to 10^7 CFU of each variant tested. The spleens of dead pups were cultured to ensure that death was a result of pneumococcal sepsis.

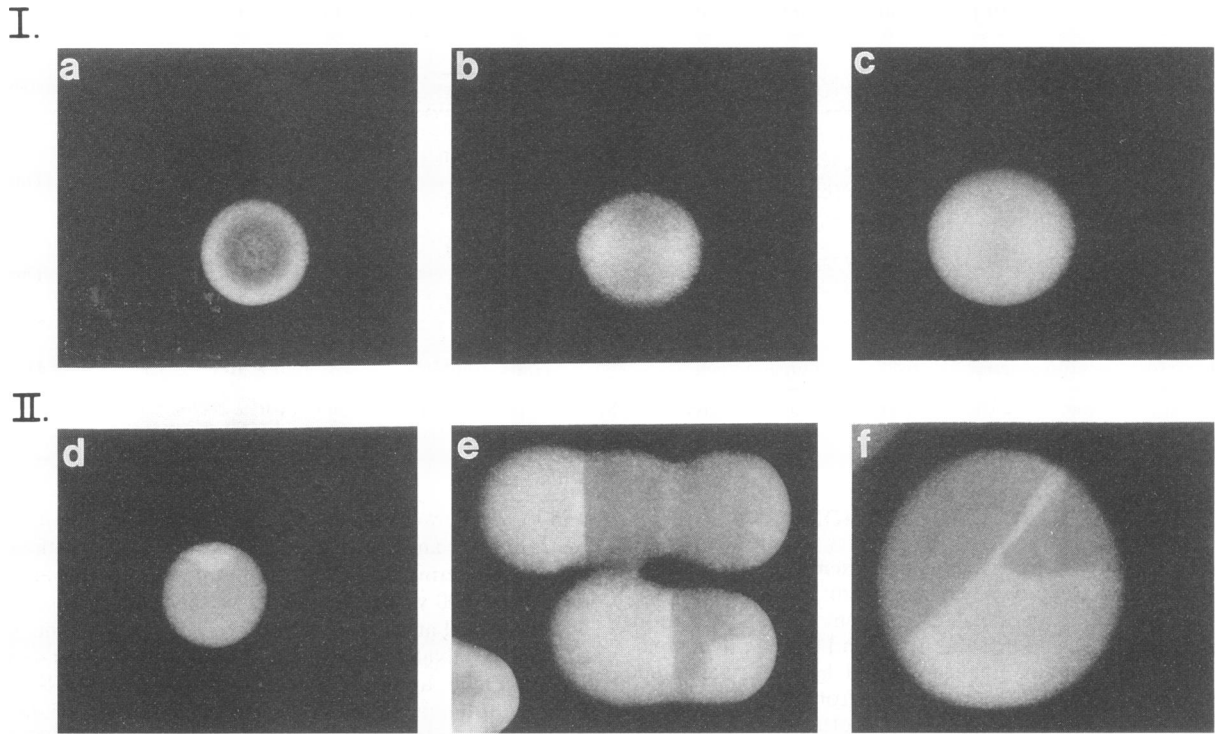

FIG. 1. (I) Photographs of individual daughter colonies derived from a single colony of a serotype 9V isolate, P62. Oblique, transmitted illumination is used to show variation in colony morphology. (a) Transparent; (b) intermediate; (c) opaque. Magnification, ×180. (II) Same technique as used in panel I showing intracolonial phenotypic variation seen as sectoring of clinical isolates of three serotypes. (d) P62 (serotype 9V); (e) P68 (serotype 18C); (f) Oliver (serotype 23F).

RESULTS

Variation in colony morphology. The pneumococcus is typically grown on nutrient agar supplemented with blood to provide a source of catalase which hydrolyzes peroxides, a normal, although toxic, product of pneumococcal metabolism (22, 23). This opaque medium allows for only a limited view of colonial morphology. The addition of catalase to transparent medium and incubation in increased levels of carbon dioxide make it possible to achieve satisfactory growth and enables the use of transmitted light to view morphologic features. Individual colonies of clinical isolates obtained from blood cultures were visualized with magnification and oblique, transmitted illumination. Variation in appearance of colonies derived from a single isolate was noted. For some isolates, at least three distinct phenotypes could be differentiated and separated into populations on the basis of variations in opacity. A typical colony of the most transparent variant appears smaller, bluish, and more transparent in the center ("bullseye") than the other colonies. The most opaque colony is slightly larger, whitish, and more uniform than the other colonies. Figure 1I shows opaque, intermediate, and transparent colonies from the same isolate of serotype 9V. When viewed with oblique, indirect illumination, the shape of a typical transparent colony is umbilicated, the intermediate opacity colony is flat, and the opaque colony is domed. Phase-contrast microscopy of the various phenotypes grown in broth cultures without agitation showed no consistent differences in average chain length or other characteristics among these variants of the same strain at a magnification of ×400. In addition, there were no significant differences among variants in the doubling time or other growth characteristics in either nutrient broth or in a semisynthetic medium.

Major differences between opaque and transparent forms on thin sections of electron micrographs were noted (Fig. 2). Plate-grown bacteria of two different serotypes were analyzed, and the differences among phenotypes were consistent for both isolates. In sections of the transparent variant of either isolate, the surface structures were less well defined, the cytoplasm appears to separate from the cell wall, and there was increased extracellular material or debris (Fig. 2B and D). This appearance is similar to that from a previous report showing pneumococcus in the process of autolysis (40). It appears, therefore, that the transparent organisms are further along in the process of autolysis than the opaque variants, although both variants were grown under identical conditions. Further studies to look at differences in autolysis showed that both the opaque and transparent phenotypes underwent lysis in prolonged culture on either agar surfaces or in broth culture, although lysis occurred somewhat sooner for the transparent variant. Both phenotypes were readily lysed in the presence of 0.1% deoxycholate. Since deoxycholate activates an autolytic amidase, this finding indicated that differences in morphology were not likely to be the result of differences in expression or function of this enzyme (25). This conclusion was confirmed by observation of opacity variation in a mutant (RUP24) with a defect in *lytA*, the major autolysin gene (41).

Characteristics of phase variation in opacity. Colonies of isolates demonstrating differences in morphology were occasionally noted to display intracolonial variation or sectoring. Figure 1II shows sectored colonies of isolates of each of three

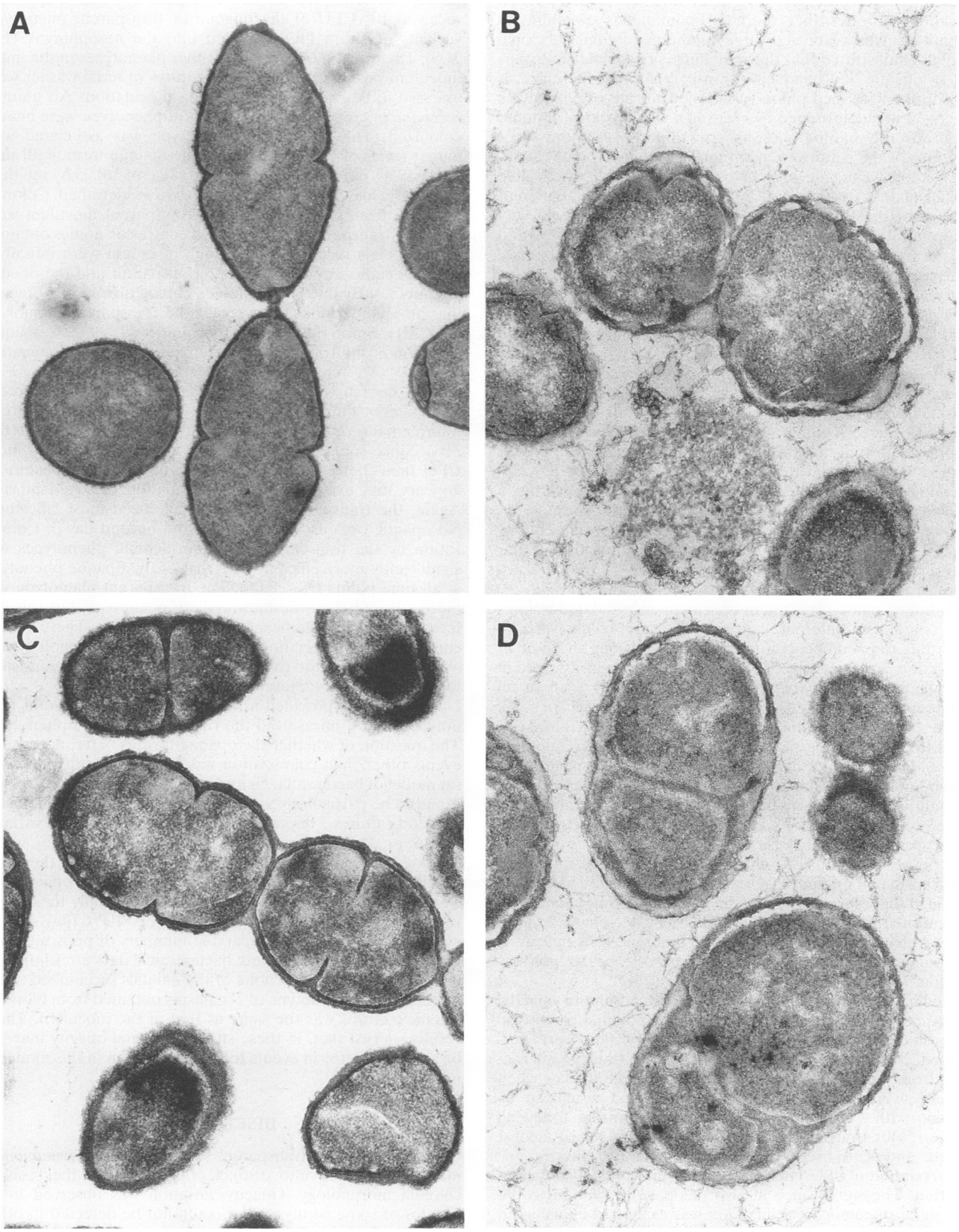

FIG. 2. Electron microscopy of thin sections of opaque and transparent variants of *S. pneumoniae* P6. (A) Opaque variant; (B) transparent variant, strain P68; (C) opaque variant; (D) transparent variant. Magnification, ×44,500 for panels A and B and ×46,500 for panels C and D.

pneumococcal serotypes, 9V, 18C, and 23F. Intracolonial variation indicates that there is spontaneous switching in phenotype. Observations of large numbers of colonies of clonal origin demonstrated that this switching is reversible (transparent to opaque and opaque to transparent) and, therefore, is appropriately termed phase variation. The rate of phenotypic reversion was determined by growing a single colony in liquid culture to the mid-log phase of growth and plating out serial dilutions of the culture to quantitate the phenotype of daughter colonies. The rate of phase variation was highly variable from isolate to isolate (10^{-3} to 10^{-6}) (Table 1). Different isolates of the same capsular type were found to have very different rates of phase variation (serotype 9V strains, P62 and P6). In general, for a single isolate, the frequencies of opaque to transparent conversion and of transparent to opaque reversion were about equal. A variety of environmental conditions, such as temperature (30 to 37°C), pH (6.8 to 8.0), osmolarity (300 to 800 mØsmol), or CO_2 level (atmospheric to 5%), did not affect the frequency of reversion.

Relationship between opacity variation and encapsulation. Opacity variants of strains P62 and P68 were compared for the presence and quantity of capsular polysaccharide. Each variant gave a positive Quellung reaction with serotype-specific antiserum, confirming that all phenotypes examined were encapsulated. The quantities of immunoreactive capsular polysaccharide associated with each variant were compared by using a whole-cell ELISA. There was no difference between the numbers of organisms of each variant required to give an equivalent ELISA reading, indicating that the quantity of capsular polysaccharide was not variable among the different phenotypes of the isolates studied.

Although phase variation in opacity in encapsulated isolates was noted, the differences in opacity were highly variable according to pneumococcal serotype. At least one strain of 27 different types was analyzed. Opacity variation was observed in the majority of serotypes but was most readily seen in all isolates of serotypes 9V ($n = 4$), 23F ($n = 6$), and 18C ($n = 3$), which are among the more common serotypes currently isolated (5). The finding of variation in colonial morphology in only a limited number of pneumococcal serotypes suggests the possibility that the presence of some capsules may obscure recognition of variations in opacity. It would be predicted, therefore, that variations in opacity would be seen in an unencapsulated mutant of a type in which it could not otherwise be observed. In fact, phase variants in the opacity of an unencapsulated mutant, P24, of a type 2 strain were identified, although differences in phenotype could not be appreciated in the capsulated parent strain, D39S (Table 1). These findings suggest that the structural basis for differences in opacity is independent of the expression and type of capsular polysaccharide.

Studies of the biochemical basis for variations in opacity. Experiments to determine if differences in colonial phenotype were associated with changes in protein profiles were performed. Membrane fractions of opaque and transparent bacteria from strain P6 showed no identifiable differences when proteins were separated by SDS-PAGE and visualized by staining with Coomassie blue or immunoblotting following Western blot transfer with polyclonal antisera raised against opaque and transparent variants of P6 (data not shown).

Correlation of differences in colonial morphology and colonization. The significance in vivo of opacity variation to the biology of pneumococcal infection was examined by using an animal model of nasopharyngeal colonization and bacteremia. Infant rats were selected as hosts because of the ease of obtaining nasopharyngeal cultures relatively free of contaminating bacteria (44). In initial experiments, 20 pups each received 10^6 CFU of the opaque or transparent phenotypic variants of strain P6 inoculated into the nasopharynx (Fig. 3IA). The fraction of variants of other phenotypes in the initial inoculum was less than 1/1,000. Cultures of nasal washes were assessed 1, 3, and 7 days following inoculation. All animals receiving organisms of the transparent phenotype were heavily colonized. The transparent phenotype was associated with higher levels of colonization than the opaque form at all time points. This difference achieved a P value of <0.05 by the third day, and by day 7, no opaque colonies were identified. Colonies obtained from pups receiving the transparent inoculum were all of the transparent phenotype. In contrast, colonies obtained from animals receiving the opaque inoculum were initially a heterogeneous population of the transparent and the opaque phenotypes. By day 7, all colonies obtained from pups receiving the opaque inoculum were of the transparent phenotype (Fig. 3II). Since the original inoculum was >99.9% opaque, selection of the transparent phenotype must have occurred in vivo.

In a separate set of experiments, 20 pups each received a smaller inoculum of 10^3 CFU of either the transparent, intermediate, or opaque variant of another strain, P68 (Fig. 3IB). Since some cultures of nasal washings contained more CFU than there were organisms in the original inoculum, it appears that expansion of the population occurred in vivo. Again, the transparent phenotype colonized most efficiently. No opaque organisms were recovered beyond day 3. Colonization by the transparent and intermediate phenotypes was significantly more efficient than that of the opaque phenotype at all time points ($P < 0.05$). The transparent phenotype was more efficient than the intermediate phenotype, but this difference was not significant at all time points. These results confirmed the association between colonial morphology and ability to colonize the rodent nasopharynx and were consistent for isolates of two different capsular types.

These results provided a function for the transparent phenotype but left unresolved the role of the opaque phenotype. The question of whether the opaque form is better adapted to events other than colonization was addressed with the infant rat model of bacteremia. Neither isolate P6 nor isolate P68 was found to be particularly virulent. Bacteremia was not detected in blood cultures 2 days after intranasal challenge with variants of P6 or P68. Following intraperitoneal challenge, which bypasses the selective pressures of the nasopharynx, there was no difference between the 50% lethal doses of the opaque and transparent forms of P6 (Table 2). However, only those pups receiving a large inoculum of strain P6 ($>10^6$ CFU) showed evidence of infection. Comparable numbers of pups receiving different variants of P68 were bacteremic 2 days after intraperitoneal inoculation, and none of the animals succumbed to the infection. The phenotype of organisms obtained from blood or splenic cultures was the same as that of the inoculum. These results suggest that, in these strains, colonial opacity may not be a critical factor in events following invasion in this model of infection.

DISCUSSION

When viewed on transparent solid medium, *S. pneumoniae* may be separated into distinct populations on the basis of colonial morphology. Opacity variation was observed in all isolates of some serotypes but could not be detected in other serotypes. It appears that some pneumococcal capsules may obscure this phenomenon, because, in at least one case, opacity variation is seen in an unencapsulated mutant but not its

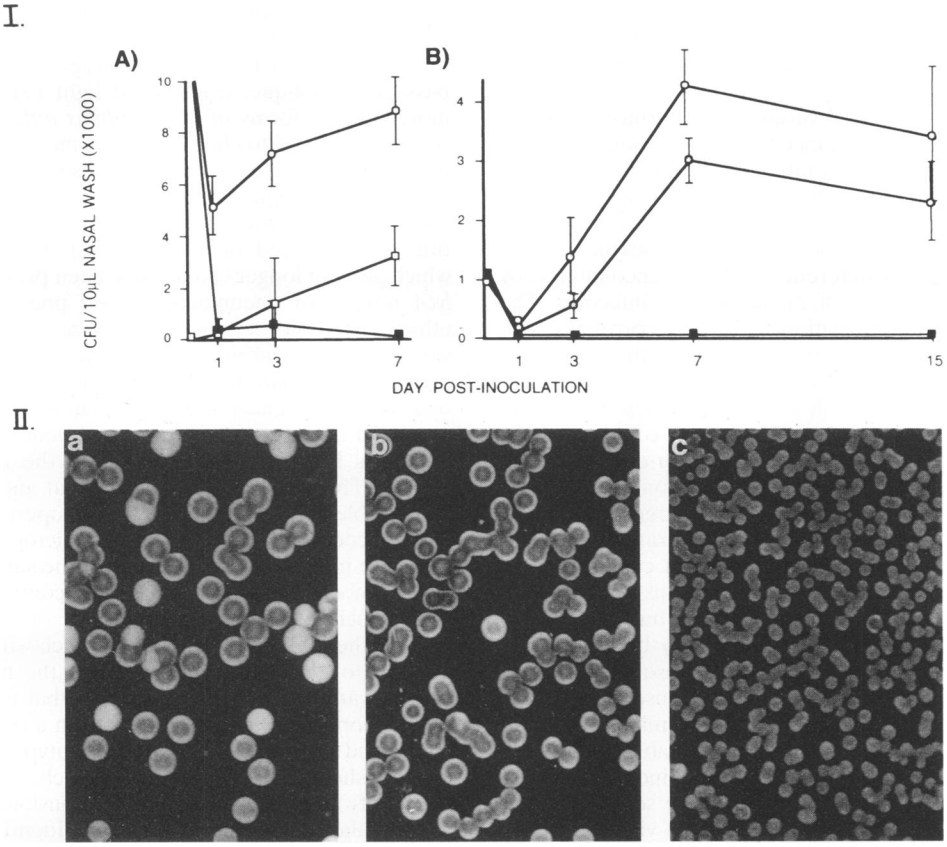

FIG. 3. Colonization of the infant rat nasopharynx by pneumococcal opacity variants. (I) Groups of 20 5-day-old infant rats each received an inoculum of transparent, intermediate (P68 only) or opaque variants of either 10^6 CFU of strain P6 (A) or 10^3 CFU of strain P68 (B). The quantity and phenotype of organisms in nasopharyngeal cultures were determined on the days following the inoculation indicated. The arithmetic mean of the number of CFU in nasal washes for each set of 20 animals receiving the same inoculum is shown. ○, transparent inoculum; ● , intermediate inoculum; ■, opaque inoculum. Colonies from cultures of nasal washes were of the same phenotype as the inoculum except for the opaque inoculum of P6. Following inoculation with the opaque phenotype of P6, organisms with both opaque (A [■]) and transparent phenotypes were obtained. The mean number of transparent variants in nasal washes from 20 pups receiving the opaque inoculum of P6 is shown graphically (A [□]) and is represented below in photographs from cultures of a single pup receiving the opaque phenotype of P6. Error bars indicate ± standard deviation (95% confidence interval). (II) Colonies from cultures of nasal washes on different days from the same pup challenged with the opaque phenotype of strain P6. Transparent variants of the opaque inoculum are present by the first day postinoculation and are the only phenotype observed by the seventh day of the experiment. (a) Day 1; (b) day 3; (c) day 7. Magnification, 60×.

parent strain. A number of the early investigators of this organism have provided descriptions of phenotypic variation associated with the pneumococcus (8, 10, 18, 28). These studies, however, relied principally on colonial morphology on the opaque surface of blood agar observed with reflected light. It is difficult, therefore, to directly compare previous observations with those in this report, which rely on a different optical technique.

TABLE 2. Intraperitoneal virulence of opacity variants in 5-day-old infant rats

Strain	Serotype	Phenotype	No. bacteremic/ no. challenged[a]	50% lethal dose (CFU)
P6	9V	Transparent		5×10^6
		Opaque		5×10^6
P68	18C	Transparent	6/8	$>10^8$
		Intermediate	4/8	$>10^8$
		Opaque	4/8	$>10^8$

[a] Determined by 10-μl blood cultures 2 days following intraperitoneal inoculation.

The varied phenotypes observed in the present investigations were not stable but underwent spontaneous and reversible phase variation. Phase variation in opacity was independent of several differences in environmental conditions. The frequency of switching of phenotype varied widely and was strain dependent. The structural basis of opacity variation remains unresolved, although several biochemical and structural features appear to be associated with these aspects of colonial morphology. Electron micrographs showed significant structural differences between phenotypes. The surface structures were less distinct, there was increased space between cell wall and plasma membrane, and extracellular debris was more prominent in the transparent forms. These findings suggest the possibility that the transparent phenotype is undergoing autolysis and, therefore, that this phenotype may have increased susceptibility to the pneumococcal autolysin. However, variation in colony morphology was shown to be unrelated to a gene responsible for autolysis. It remains possible that differences in

the cell wall contribute to opacity variation. Intrastrain differences in cell wall antigenicity have been demonstrated and indicate that there is structural heterogeneity in the cell wall polysaccharide (32).

The observation of phase variation in surface structure suggested the potential significance for this phenomenon in the pathogenesis of pneumococcal infection, as has been demonstrated for other pathogenic bacteria that occupy a similar environmental niche (19, 44). A further implication of the observation of phase variation is that each phenotype conveys a selective advantage for different challenges encountered by the organism in the course of pneumococcal infection. The results demonstrate that variation in opacity correlates with differences in the interaction of organisms with a host. An animal model was used to show that only the transparent phenotype was able to establish a state of efficient and stable colonization of the nasopharynx. This result could not have been attributed to differences in growth rate or encapsulation. As further evidence for the association of colonial phenotype with colonization, it was shown that when a large inoculum of opaque organisms (P6) was administered intranasally, transparent variants were recovered in subsequent cultures of the nasopharynx. In contrast, the opaque organisms of a different strain (P68) were deficient in colonization, but transparent variants were not detected. This finding may be due to the lower rate of spontaneous variation in this isolate. Alternatively, the inoculum, which consisted of a thousandfold fewer bacteria, may have contained an insufficient number of variants of the transparent phenotype to become established. In the case of strain P6, either the original inoculum included a small number of transparent organisms which were selected in the nasopharynx or phase variation occurred in vivo. In either instance, selection of the transparent phenotype in preference to the opaque phenotype led to marked expansion of the transparent population and clearance or death of the opaque organisms.

A potential role for the opaque phenotype in events following nasopharyngeal colonization in invasive infection was tested. The animal model utilized, however, failed to reveal a biological role for the opaque phenotype. It is known that pneumococcal types vary widely in their virulence in rodents (46). The strains of pneumococcal types examined in these experiments were selected because of features of their colonial morphology and have relatively low virulence in rodents (24). Similar studies with opacity variants of strains with greater virulence in rodents may be more revealing.

Phase variation apparent as differences in colonial opacity has been noted previously for streptococci other than the pneumococcus. McCarty reported spontaneous opaque variants of group A streptococci; these variants form extremely long chains compared with those of the more transparent forms (21, 37). The biological significance of these differences in colonial morphology has not been examined. Recently, Pincus et al. reported opaque variants of group B streptococci, which also grow in longer chains (31). Differences in the growth, virulence, and immune response to these opacity variants have been described, while the relationship of phenotypic variation to the pathogenesis of group B streptococcal infection remains unknown (30). Observations in this report on *S. pneumoniae* differ from those of the other streptococcal species in a number of important aspects. No differences in either chain length, encapsulation, or growth characteristics were associated with differences in pneumococcal opacity. Characteristics of the pneumococci from transparent colonies in electron micrographs in this report were not seen in variants of either group A or group B streptococci (31, 37).

In some bacterial species, it has been shown that the relative opacity of a colony is a consequence of how closely the organisms within the colony aggregate and its effect on the passage of oblique, transmitted light (9). Organisms of the more opaque forms of *Haemophilus influenzae* and *Neisseria gonorrhoeae* tend to clump together more than the transparent forms (35, 44). This relationship also appears to explain why longer chain length associated with groups A and B streptococci leads to tighter association of cells within a colony and, thus, to enhanced opacity (21, 31). Pneumococcal variants which grow in longer chains have been previously identified. A *lytA* mutant of pneumococcus and pneumococcus grown in ethanolamine form long chains (38, 39). Also, filamentous variants from marginal excrescences of colonies following prolonged incubation have been reported (3). In contrast, the opacity of the encapsulated pneumococci examined in this study did not appear to be a consequence of increased chain length as has been documented for the other streptococcal species. This observation indicates that another mechanism is responsible for the altered optical properties of organisms in pneumococcal colonies. Electron micrographs showed that there are major differences between the surface characteristics of pneumococcal variants, which may contribute to how organisms associate within a colony.

In further studies, a genetic approach will be followed in an attempt to identify and characterize the molecular basis for opacity variation. It has been found that total genomic DNA from an opaque variant will transform a transparent recipient strain and confer the opaque phenotype at high frequency (unpublished data). With this approach, it should be possible to identify a chromosomal locus responsible for the expression and regulation of colonial opacity. Identification of such a locus would facilitate the generation of defined mutants to examine further the contribution of opacity variation to the biology and pathogenicity of *S. pneumoniae*.

ACKNOWLEDGMENTS

We thank E. Tuomanen for critical review of the manuscript. The *lytA* mutant strain was generously provided from the collection of A. Tomasz. Clinical isolates were provided by Karin McGowan.

J. N. Weiser is a Lucille P. Markey Charitable Scholar. This work was supported by grants from the Lucille P. Markey Charitable Trust (J.N.W.), I. T. Hirschl Trust (H.R.M.), and Public Health Service (RO1-A127913 and R29-A132024 [H.R.M.]).

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