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Expression of M Type 12 Protein by a Group A Streptococcus Exhibits Phaselike Variation: Evidence for Coregulation of Colony Opacity Determinants and M Protein

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Three major categories of colony opacity were observed for natural variants of the M type 12 (M12) group A streptococcus strain CS24. Colony opacity variants that switched between two alternative categories at significantly high frequencies were identified and are referred to as switching between more opaque (Op^+) and less opaque (Op^-) phenotypes. Twenty lineages of such variants were derived for analysis and were assessed for resistance to phagocytosis, acid-extractable M12 antigen, and M12 mRNA, criteria which define the M protein-positive phenotype (M^+). Transition from the M^+ to the M protein-negative phenotype (M^-) correlated with a change from Op^+ to Op^-. Reversion to the Op^+ phenotype was accompanied by reversion to the M^+ state in all variants except one and occurred at a higher frequency than the forward M^+ to M^- switch. These data demonstrate the existence of M12 protein phaselike switching in the group A streptococcus strain CS24. The discovery of an Op^+ M^- revertant confirmed that colony opacity and M protein can be expressed independently and are distinct gene products. We suggest that coregulation of colony opacity and M protein expression accounts for their association among descendents of strain CS24. Southern blot hybridization analyses of digested genomic DNA from 27 M^- variants and 15 M^+ revertants were performed with DNA probes containing M12 protein and adjacent upstream sequences. DNA deletions were identified only in two stable M^- variants, approximately 1.3 and 1.4 kilobases upstream from the M12 gene, respectively, whereas all unstable M^- variants lacked detectable rearrangements. This suggests that deletions within or adjacent to the structural gene are unlikely to be responsible for the reversible switch in M protein expression. However, the association with the stable M^- phenotype and the location of these deletions, as well as two other deletions, approximately 0.5 kilobase upstream from the M12 promoter in two previously described variants of strain CS24 suggests that a second gene product is required for full expression of M12 protein synthesis in this strain.

Streptococcus pyogenes, the causative agent of human streptococcal pharyngitis, impetigo, and other, more serious maladies, produces a variety of macromolecules which contribute to its ability to circumvent the immunological defenses of its host. M protein is associated with hairlike fibers on the cell surface (28) and is known to impede opsonization by the alternative complement pathway (2), a requirement for efficient ingestion by polymorphonuclear leukocytes. Currently there are over 80 immunologically distinct M protein antigens recognized (6), and evidence suggests that such diversity is the result of antigenic drift (16).

Although instability of the M protein-positive phenotype (M^+) upon cultivation of clinical isolates on laboratory media or after prolonged carriage by patients (21) has been observed, the genetic basis of this instability is only beginning to be understood (26). Variants that are sensitive to phagocytosis and fail to produce detectable levels of M antigens (M^-) are easily isolated from most strains. Several bacterial pathogens including *Neisseria gonorrhoeae* (27) and *Escherichia coli* (5) express surface antigens that exhibit reversible switching at high frequency between the positive and negative phenotype, instability that is referred to as phase variation. Although we have suggested that M protein similarly undergoes phase switching (25, 26), a systematic analysis of this phenomena is lacking.

It was discovered in our laboratory that a bacteriophage was required by a spontaneous M^- variant of an M type 76 strain for reversion to the M^+ phenotype (23). This phage, however, has no affect on M^- variants from other strains, including M^- variants of the M type 12 (M12) strain CS24 from which it was derived (unpublished observation). More recently, analysis of two independent spontaneous M^- variants of strain CS24 identified small deletions located at nearly identical loci within a 1.2-kilobase (kb) fragment containing the M12 gene (26). These results suggested that deletion formation was an important component of genetic instability and questioned whether bacteriophage genes have a general impact on M protein expression.

The primary goal of this study was to determine whether group A streptococci switch between M^+ and M^- states at frequencies high enough to be characteristic of true phase variation, or whether cultures merely accumulate mutations which interfere with the M^+ phenotype. Secondary to this question is whether specific DNA rearrangements within or adjacent to the M12 gene, such as deletion formation, accompany the switch between the M^+ and M^- phenotypes. To address these questions, it was necessary to isolate numerous independent M^- variants. This required a simple method to distinguish between colonies of M^+ and M^- cells on agar. Griffith (9) reported the isolation of type-specific M^+ strains by selecting opaque colony variants, and Gooder and Maxted (7) later suggested that the opaque nature of colonies reflected a high content of M protein. However, colony opacity determinants and M protein have been shown to be distinct characteristics (29) and are frequently expressed independently of each other (17, 32). Despite this, more recent work has suggested that colony opacity as a marker for M expression is strain variable (22). In this study, we

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TABLE 1. Summary of phase variant characteristics

Variant designation	Opacity phenotype	Reversion frequency[a]	Detection of M12 protein expression		Resistant to phagocytosis
			Antigen[b]	mRNA (%)[c]	
A3, A7, A9, A13	Op^-	$<10^{-6}$	−	0	No
A1	Op^-	10^{-2}–10^{-3}	−	0	No
A2, A4, A5, A6, A14, A15, A17, A19, A20	Op^-	10^{-2}–10^{-3}	+	1	No
A8, A10, A11, A12, A16, A18	Op^-	10^{-1}–10^{-2}	+ +	10	No
B1	Op^+	NA[d]	−	0	No
B2, B4, B5, B6, B8, B10, B11, B12, B14, B15, B16, B17, B18, B19, B20	Op^+	NA	+ + +	100	Yes
C18	Op^-	$<10^{-6}$	−	0	No
C14, C15, C16, C17, C19	Op^-	10^{-2}–10^{-3}	+	1	No

[a] B-series cultures segregated Op^- colonies at a frequency of between 10^{-3} and 10^{-4}.

[b] Presence of immunoprecipitin arc with acid extracts as the antigen source: −, negative; +, weak positive; + +, positive; + + +, strong positive.

[c] Quantity of mRNA detected in total RNA from M protein phase variants is indicated as the percentage of M12 mRNA detected in total RNA from strain CS24.

[d] NA, Not applicable.

report that M^- variants of the M12 strain CS24 generally form colonies which have an opacity distinctly different from that of colonies formed by M^+ cells. This difference in colony morphology offered a reliable means of monitoring switching between the M^+ and M^- state and enabled the successful isolation of M protein phase-switching variants for genetic analysis. Surprisingly, DNA from all M protein switching variants had no evidence of rearrangements within or adjacent to the M12 gene. Deletions like those previously described (26) were not identified, but two of five phase-locked M^- variants had deletions at different loci upstream of the M12 gene. Our results suggest that although these deletions may contribute to the formation of the stable M^- state, they are unlikely to be responsible for phase variation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM83 carrying the plasmid pPC101 and the M12 group A streptococcus strain CS24 were obtained from laboratory stocks and have been described previously (3, 26). Strain JM83 carrying the vector pUC9 (31) with the *Hae*III A fragment of pPC101 as an insert was obtained from laboratory stocks. This hybrid plasmid is designated as pPC124 and expresses an M12 antigen identical to that produced by the parent streptococcal strain CS24 (J. C. Robbins, J. G. Spanier, S. J. Jones, and P. P. Cleary, submitted for publication).

All opacity phase variants once isolated were confirmed to be group A streptococci by the capillary precipitin test as previously described (13) and were then stored in Todd-Hewitt broth at −70°C. These variants were assigned strain designations consisting of A, B, or C depending on their series and a number between 1 and 20 corresponding to their lineage. Opacity phase variants are listed in Table 1. Strains CS46 and CS64 have been previously described (26).

Culture media. Liquid cultures were routinely propagated in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) plus 1% (wt/vol) neopeptone (Difco). This medium was prepared immediately before use to minimize aeration, and cultures were incubated at 37°C for 16 h. Log-phase cells were prepared by inoculating (5% [vol/vol]) fresh Todd-Hewitt broth from 16-h-old broth cultures and incubating the cells for 3 to 4 h at 37°C. Islam medium was prepared as previously described (22).

Colony opacity determination. Colonies were cultured anaerobically (GasPak [H_2-CO_2]; BBL Microbiology Systems, Cockeysville, Md.) on Islam medium at 37°C. After 18 h of incubation, colony morphology was examined with a stereomicroscope (model 205819; Nikon) with a blue 80B Kodak wratten gelatin filter and obliquely transmitted light. Colonies were photographed with a 35-mm camera and Kodak ASA 400 film.

The frequency at which more opaque (Op^+) colonies segregated less opaque (Op^-) colonies and vice versa was estimated by a modification of the method described by Eisenstein (5). Blocks of agar containing single colonies were removed from the plate and separately vortexed for 30 s in 1 ml of sterile Todd-Hewitt broth. Samples of 50 μl from 10-fold dilutions of bacterial suspensions were then dispensed onto Islam medium by the method of Miles and Misra (18) so that viable counts of the Op^+ and Op^- cells within the colonies could be determined.

M protein assays. M12 protein acid extracts from 100-ml cultures were prepared as described by Lancefield (12) and assayed by double immunodiffusion with M12 antiserum (23). Cells were tested for resistance to phagocytosis by their growth in heparinized whole human blood by the method of Lancefield (13) with modifications as described in the Results section.

Quantitation of M12 mRNA. Cells from late-log-phase 100-ml cultures (10^8/ml) were resuspended in Todd-Hewitt broth (pH 6.1) supplemented with 30% (wt/vol) sucrose, 0.5 mM magnesium chloride, and 0.5 mM dithiothreitol, and they were treated with group C streptococcal phage-associated lysin to form protoplasts (J. C. Robbins, personal communication). Total RNA was extracted in the presence of 4 M guanidium isothiocyanate (14) and separated from DNA by ultracentrifugation in a 5.7 M cesium chloride cushion (8). The RNA pellet was suspended in RNase-free distilled water. The M12-specific probe was a 0.54-kb fragment from within the M12 gene at position 0.49 to 1.03 kb (see Fig. 7). This fragment was derived from plasmid pPC101 as a phage M13mp18 subclone and was prepared as a strand-specific M13 probe by the method of Hu and Messing (11). Northern (RNA) blots performed with formaldehyde agarose gels as described by Maniatis et al. (14) indicated that this probe was specific for group A streptococcal RNA transcripts of 2 kb in length (Robbins et al., submitted). To compare the quantity of M12 mRNA produced by Op^+ and Op^- cells, total RNA was spotted onto nitrocellulose and probed with the M12-specific probe by the method of Thomas (30).

DNA analysis. Genomic DNA was prepared as previously described (24), with the exception that protoplasts were prepared by digestion with mutanolysin (100 μg/100 ml of culture). After pronase treatment, the DNA was extracted twice with 1 volume of phenol, twice with phenol–chloroform-isoamyl alcohol (1:1, vol/vol), once with chloroform-isoamyl alcohol (24:1, vol/vol), and four times with ether. After this, 0.1 volume of 3 M sodium acetate (pH 6.0) and 2 volumes of ethanol were added to the extract, and the

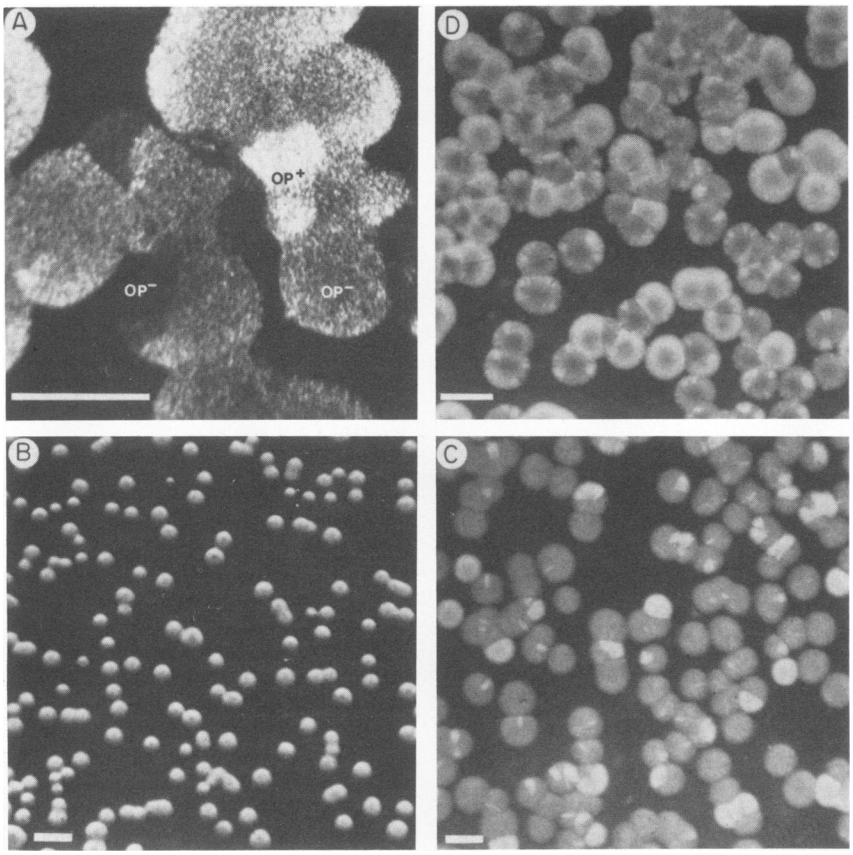

FIG. 1. Colony morphology of phase variants of strain CS24 as viewed by light microscopy with obliquely transmitted light. (A) Different degrees of colony opacity corresponding to the wild-type Op^+ and two less opaque (Op^-) colony types. (B and C) Colonies formed from cells cultured either aerobically (B) or anaerobically (C). Note the small and uniform opaque colonies formed aerobically compared with the larger, opacity-variable colonies formed anaerobically. (C and D) Comparison of the extent of Op^+ sectoring in Op^- colonies of variants exhibiting reversion frequencies of 10^{-1} to 10^{-2} (D) and 10^{-2} to 10^{-3} (C). Bars, 1.5 mm.

precipitated DNA was spooled onto a glass rod, rinsed in 70% alcohol, and suspended to a final concentration of 0.5 μg/μl in distilled water.

Plasmid DNA was extracted from 500-ml 16-h-old cultures containing 50 μg of ampicillin per ml by a previously described cell lysis method (4). After centrifugation of the cell lysate at 35,000 × *g* for 40 min, 0.1 volume of 2 M sodium chloride was gently mixed into the supernatant and the preparation was heated at 70°C for 15 min. The precipitate was removed by centrifugation (12,000 × *g*, 10 min at 4°C), and the supernatant was treated with RNase and extracted with phenol–chloroform-isoamyl alcohol (1:1, vol/vol). Plasmid DNA was ethanol precipitated and purified by two consecutive cesium chloride-ethidium bromide density gradients (19), employing 220,000 × *g* for 8 h at 20°C in a Beckman VTi 65 rotor.

Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.), and digestions were performed as recommended by the manufacturer. Electrophoresis of DNA was in 0.7 or 1.5% agarose gels at 50 V for 15 h. The methods of transferring DNA from agarose gels to nitrocellulose, high-stringency hybridization, and autoradiography have been described previously (24).

Plasmid pPC101 and pPC124 probes were radiolabeled in vitro with [α-^{32}P]dCTP by nick translation according to the directions of the manufacturer (nick translation reagent kit; Bethesda Research Laboratories). Phenol was distilled and saturated with STE buffer (20 mM Tris [pH 8.0], 1 mM EDTA, 100 mM sodium chloride) before use, and chloroform was mixed with isoamyl alcohol (24:1, vol/vol). All chemicals were analytical reagent grade.

RESULTS

Colony opacity variation. For colony opacity to be reliably used as a marker for monitoring changes in M protein expression in strain CS24, colony morphology was thoroughly defined. M^+ cultures of strain CS24 produced predominately opaque colonies (Op^+), but regularly segregated two morphologically distinct less opaque (Op^-) colony types at high frequency (Fig. 1A). Both Op^- colonial types reverted to the more opaque (Op^+) wild-type morphology. The different degrees of colony opacities observed in many Op^- variants cannot be explained at this point, but these variants proved to be similar with regard to M protein expression. Differences corresponding to Op^+ and Op^- colony morphologies were observed only when cultures were incubated anaerobically on Islam medium (Fig. 1B and C).

Op^+ colonies segregated Op^- colonies at a frequency of between 10^{-3} and 10^{-4}. Op^+ revertants were recovered at frequencies of between 10^{-1} and 10^{-2} or between 10^{-2} and 10^{-3}, depending on the specific lineage (Fig. 1C and D; Table 1). Some Op^- variants appeared stable as they were not observed to revert to the original Op^+ phenotype.

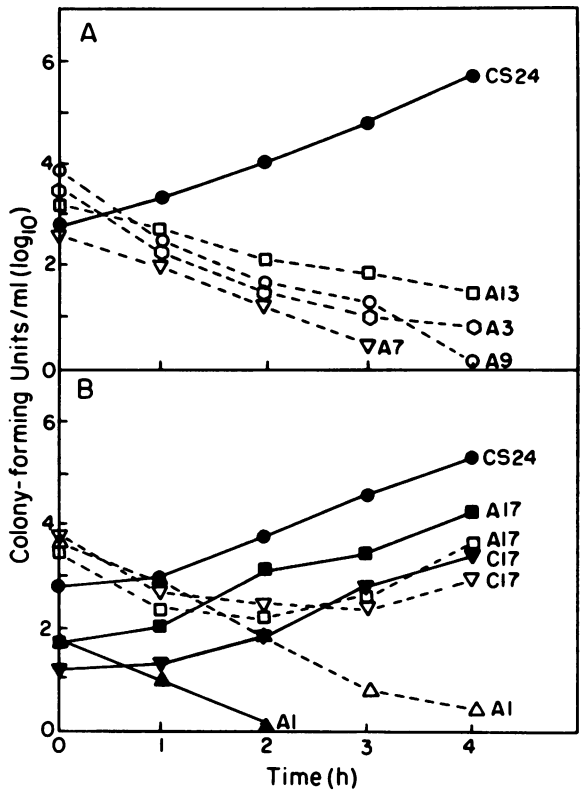

FIG. 2. Susceptibility of opacity variants to phagocytosis. Log-phase cells were diluted 10^3 to 10^5/ml in distilled water, and 0.2 ml was added to 1.8 ml of human blood. This mixture was then rotated at 6 rpm at 37°C, and samples were withdrawn at intervals for viable counts. Viable counts were determined on Islam medium, and because some variants were mixtures, colonies were counted as either Op^+ or Op^-. Survival profiles of stable Op^- cells (A) and unstable Op^- cells and their Op^+ revertants (B). Open symbols: Op^- CFU; filled symbols: Op^+ CFU.

Isolation of independent colony variants. To isolate M protein phase variants for genetic analysis, 20 lineages of colony opacity phase variants were derived as follows and subsequently tested for M12 expression. Cells from 20 wild-type strain CS24 Op^+ colonies were transferred seven times to ensure independence of each parent Op^+ line. With each passage, only Op^+ colonies having no visible Op^- sectors were selected. After passage 7, the subsequent isolates represented 20 Op^+ wild-type lineages, numbered 1 to 20. Cells from Op^- colonies were also subcultured seven times on Islam medium. These isolates were then stored and constituted series A. Similarly, Op^+ revertants were derived from most lines of the A series, and these were designated series B. Variants A3, A7, A9, and A13 were stable with respect to colony opacity and were therefore termed phase locked. To analyze variants that passed through a second phase change, we also similarly derived six Op^- variants from sectors of series B colonies, representing lines 14, 15, 16, 17, 18, and 19; these constituted series C. Variant C18 was phase locked and therefore did not yield Op^+ revertants. The B-series cultures yielded Op^- colonies at the expected frequency of between 10^{-3} and 10^{-4}.

Susceptibility of Op^+ and Op^- variants to phagocytosis. Opacity phase variants described in the previous section were assessed for their resistance to phagocytosis. This is one of several criteria used to define the M^+ phenotype. All Op^+ variants (B series), with the exception of variant B1, survived blood rotation and exhibited growth rates similar to that of the Op^+ wild-type strain CS24. Transition of the 20 parent Op^+ lines to the Op^- state (series A) produced streptococci that were susceptible to phagocytosis. Stable Op^- variants (Fig. 2A), however, exhibited a different survival profile from that seen for unstable Op^- variants (Fig. 2B).

The stable Op^- variants A3, A7, A9, A13 (Fig. 2A), and C18 (not shown) were phagocytized when incubated in nonimmune human blood, and no survivors were detected after 4 h of incubation. In contrast to these cultures, unstable Op^- variant cultures always contained small numbers of Op^+ cells which proliferated throughout the incubation period. By routinely quantitating survivors of phagocytosis on Islam medium, it was possible to contrast the survival of Op^+ and Op^- variants in cultures which were persistently a mixture of the two phenotypes. The Op^- cells in these cultures were phagocytized at the same rate as stable Op^- cells during the first 1 to 2 h of incubation, e.g., Op^- cells in variant A17 and C17 cultures (Fig. 2B). Although some unstable Op^- cells survived and began to proliferate after 2 h of incubation in blood, these survivors exhibited the same initial susceptibility to phagocytosis on reexposure to fresh human blood.

This indicates that Op^- survivors did not express antiphagocytic determinants and suggests that the accumulation of Op^+ cells retards the phagocytosis of Op^- cells. To test this possibility, stable Op^- cells from A13 cultures were mixed with viable Op^+ cells, strain B18, and then tested for sensitivity to phagocytosis. Under these conditions, Op^- cells resisted phagocytosis and showed growth kinetics similar to those of Op^- cells in cultures of strains A17 and C17 (Fig. 3). Understanding the mechanism by which viable Op^+ cells retard the phagocytosis of Op^- cells is of interest but is beyond the scope of this study.

To eliminate the possibility that survival of Op^+ cells, in contrast to Op^- cells, reflects differences in growth rate in human blood rather than susceptibility to phagocytosis, we did a series of phagocytosis experiments in the presence and absence of cytochalasin B (Fig. 4), a known inhibitor of

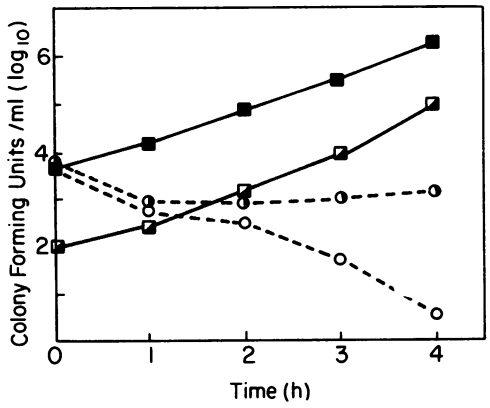

FIG. 3. Influence of Op^+ cells on the phagocytosis of stable Op^- cells. A mixture of Op^- (strain A13) and Op^+ (strain B18) cells at a ratio of 20:1 was diluted in distilled water to 8×10^4 CFU/ml. A 0.2-ml sample of this bacterial suspension was then added to 1.8 ml of whole human blood, and the phagocytosis experiment was performed as described in the legend to Fig. 2. Controls included Op^- cells only and Op^+ cells only, each diluted in distilled water to 8×10^4 CFU/ml. Symbols: Op^- CFU (◑) and Op^+ CFU (◪) in the A13 and B18 cell mixture; ○, Op^- CFU only; ■, Op^+ CFU only.

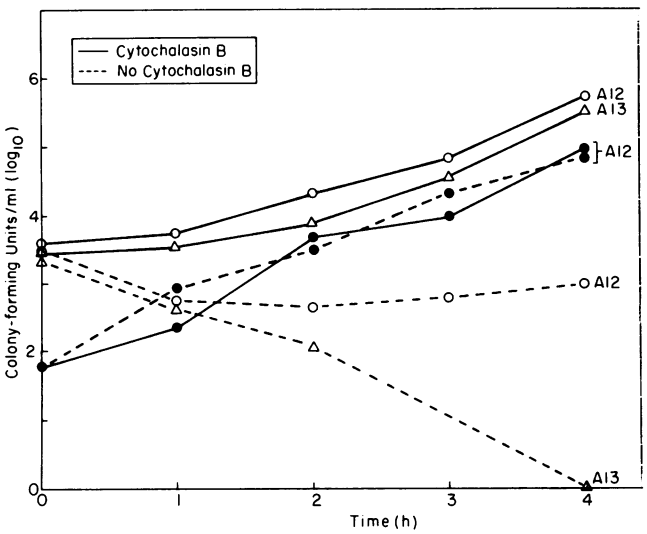

FIG. 4. Effect of cytochalasin B on elimination of Op⁻ variants from blood. Log-phase cells were diluted in distilled water to 5×10^4 CFU/ml, and 0.2 ml was added to 1.8 ml of human blood containing 20 μg of cytochalasin B per ml. Before inoculation, blood containing cytochalasin B was left at room tmeperature for 30 min to inactivate the polymorphonuclear leukocytes. Controls were untreated samples of the same blood inoculated with 0.2 ml of bacterial suspensions. Phagocytosis experiments were performed as described in the legend to Fig. 2. Symbols: ○, △, Op⁻ CFU; ●, Op⁺ CFU.

phagocytosis (33). In cytochalasin B-treated blood, Op⁻ cells from both unstable variant A12 and stable variant A13 cultures had growth rates similar to those of phagocytosis-resistant Op⁺ revertants (Fig. 4). This confirms that the initial decrease in Op⁻ cells relative to Op⁺ cells in mixed cultures is the consequence of phagocytosis.

Expression of M12 antigen by Op⁺ and Op⁻ variants. Acid-extractable M12 antigen was recovered from all Op⁺ B-series isolates, except B1 (Table 1). Although lesser amounts of antigen were recovered from unstable Op⁻ variants, it was clear that isolates having a higher reversion frequency gave stronger immunoprecipitin arcs than those with a lower reversion frequency (Table 1). Detection of

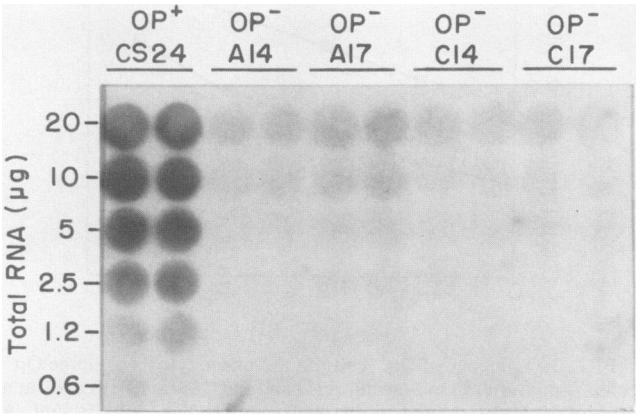

FIG. 5. Quantitation of M12-specific mRNA in Op⁺ and Op⁻ variants. Serial dilutions of total RNA were spotted onto nitrocellulose in duplicate and probed with [α-^{32}P]dATP-labeled DNA internal to the M12 gene, fragment 0.49 to 1.03 kb (see Fig. 7). Hybridization signals were eradicated when RNA preparations were treated first with RNase which was free of DNase (10 μg/ml for 1 h).

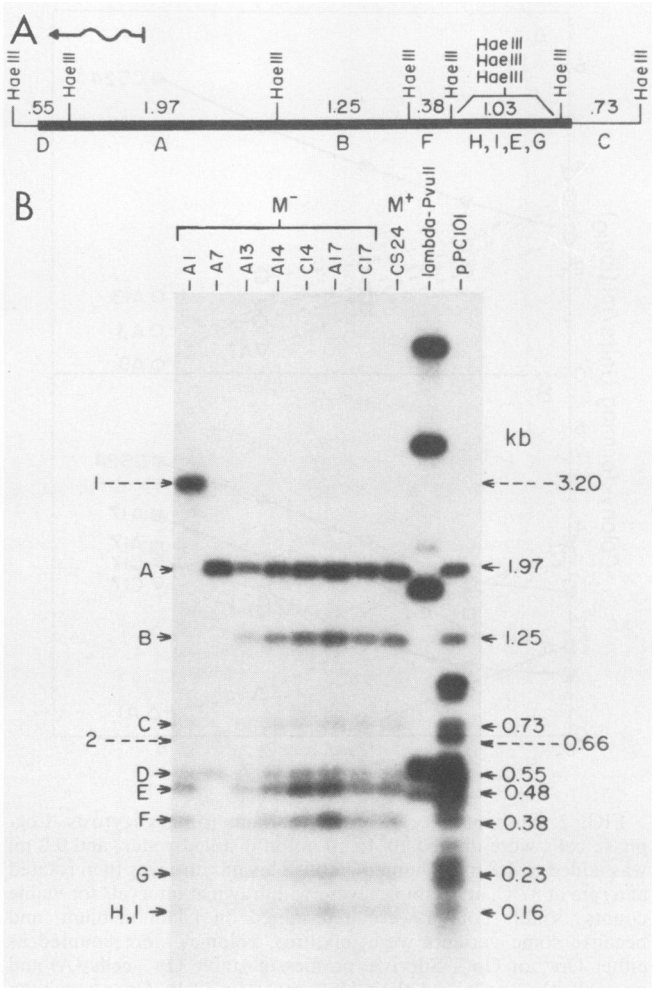

FIG. 6. Hybridization of pPC101 probe to *Hae*III restriction endonuclease digests of genomic DNA from M⁻ phase variants of strain CS24. (A) *Hae*III restriction map of DNA which hybridizes to plasmid pPC101. The region corresponding to cloned streptococcal DNA in pPC101 is shown schematically as the thick line. The arrowed wavy line represents the location and direction of the M12 protein gene. (B) Autoradiograph of *Hae*III-digested DNA probed with plasmid pPC101 DNA. Digested DNA was subjected to electrophoresis on a 0.7% agarose gel at 50 V for 15 h and then Southern blotted and probed with ^{32}P-labeled pPC101. Molecular weight markers were *Pvu*II-digested lambda DNA fragments (lambda-PvuII); the dotted arrows 1 and 2 indicate unique fragments not seen in the strain CS24 DNA.

antigen in extracts of unstable Op⁻ variants is, therefore, attributed to their mixed condition. M12 antigen was not detected in extracts of phase-locked Op⁻ variants A3, A7, A9, A13, or C18. Similarly, M12 antigen was not detected in extracts of the unstable A1 culture or an Op⁺ revertant, strain B1.

Quantitation of M12-specific mRNA produced by Op⁺ and Op⁻ variants provided further evidence that the switch from the Op⁺ to the Op⁻ state parallels a loss or significant reduction in the expression of the M12 gene. To detect M12 message, we probed total RNA with a radiolabeled DNA probe internal to the M12 gene (0.49 to 1.03 kb) (see Fig. 7). All unstable Op⁻ variants had 10- to 50-fold less M12 mRNA than the M⁺ strain CS24 (Fig. 5). We assume that low levels of M12 mRNA in unstable Op⁻ variants are the product of

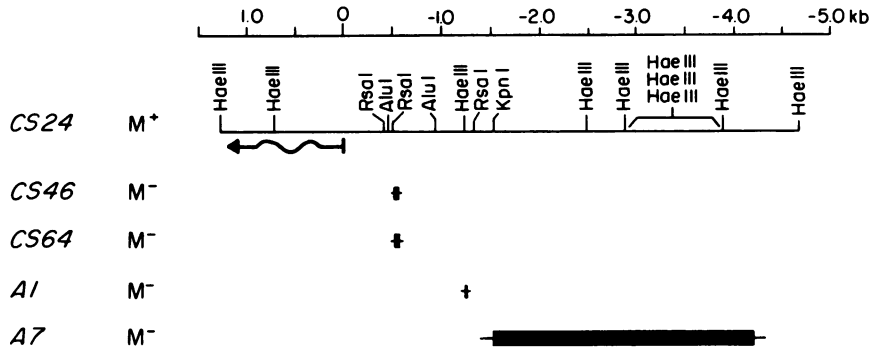

FIG. 7. Comparison of stable M⁻ phase variant DNA alterations. The location and approximate size of deletions in M⁻ phase variants are indicated by solid blocks below the restriction map; the thin lines denote their approximate boundaries. Not all *Rsa*I or *Alu*I sites are indicated.

Op⁺ revertants in these cultures. M12 message was not detected in stable Op⁻ variants (data not shown). As expected, Op⁺ revertants that were resistant to phagocytosis exhibited levels of M12 message (data not shown) equivalent to that produced by the parent strain CS24. These results suggest that transition to the M⁻ phenotype is the result of a genetic event which either blocks transcription or shortens the half-life of M12 mRNA.

Genetic analysis of M protein phase variants. On the basis of data presented in the previous sections, and with the exception of variant A1 and its Op⁺ revertant, strain B1, we conclude that all Op⁺ variants (strain CS24 and series B isolates) are resistant to phagocytosis and express M12 antigen (M⁺), while all Op⁻ variants (series A and C isolates) are susceptible to phagocytosis and express either little or no M12 antigen (M⁻) (Table 1). Because other M protein phase variants were reported to have small deletions (24), the variants isolated here were examined for deletions to determine whether deletion formation plays an integral role in phase switching.

Purified chromosomal DNA from 20 A variants, 15 B variants, and 6 C variants was separately digested with *Alu*I, *Hae*III, or *Rsa*I and then analyzed by gel electrophoresis. Southern blot hybridizations were performed under high stringency with the plasmid pPC101 as the probe (26). Plasmid pPC101 contains a 4.8-kb streptococcal DNA insert which includes the M12 coding sequence (Fig. 6A). All phase variants with the exception of M⁻ phase-locked strains A1, B1, and A7 when compared with the parental strain CS24 had no detectable differences in *Hae*III (Fig. 6B), *Alu*I, or *Rsa*I fragments (data not shown). Variant A1 lacked both the *Hae*III A and B fragments, but contained a new larger fragment (3.2 kb) (Fig. 6B, arrow 1). This fragment is approximately equal in size to the sum of *Hae*III A and B. Furthermore, *Alu*I, *Rsa*I, *Kpn*I, or *Hpa*I fragments were not affected by this alteration (data not shown), suggesting that a deletion of less than 50 base pairs had eliminated the *Hae*III A and B junction at position −1.3 kb (Fig. 7). The Op⁺ variant, strain B1, retained the deletion carried by its Op⁻ parent, strain A1 (data not shown). Strain A7 harbored a large deletion which eliminated the *Hae*III B, F, H, I, E, G, and C fragments. Southern blot analysis of *Rsa*I, *Hpa*I, *Kpn*I, and *Alu*I chromosomal digests of strain A7 with both pPC101 and pPC124 (contains only the *Hae*III A fragment of pPC101) as probes indicated that approximately 3 kb of DNA between coordinates −1.4 and −4.4 kb had been deleted (Fig. 7).

M⁻ variants CS46 and CS64, detected in an earlier study by their glossy colony morphology, contain a 50-base-pair deletion internal to *Hae*III-A (26). Southern blot data (not shown) with pPC124 as the probe showed that deletions in these variants had eliminated the *Rsa*I site at position −0.5 kb (Fig. 7). Therefore, as these deletions are considerably closer to the M12 gene than those carried by variants A1 and A7 (Fig. 7), deletions associated with an M⁺ to M⁻ switch are not necessarily confined to a specific locus.

DISCUSSION

This study established that expression of the M12 protein by group A streptococcus strain CS24 undergoes true phase variation. Although the biochemical nature of the M protein switch is unknown at present, data presented indicate that transcriptional control is involved. Somewhat analogous to phase switching of pilin and outer membrane protein II expression in *N. gonorrhoeae* (27), switching between M⁺ and M⁻ phenotypes could be monitored by changes in colony phenotype. Variation from Op⁺ to Op⁻ colony morphology coincides with transition from the M⁺ to the M⁻ state, while reversion to the Op⁺ phenotype returns the culture to an M⁺ state. These results support the early observation of Griffith (9) that colony opacity is a useful marker for M protein expression and are in agreement with Skjold and Wannamaker (22) who identified M⁺ and M⁻ variants of type 49 streptococci isolated in the United Kingdom by their colony opacity phenotype. The association of colony opacity type with M protein expression may vary from strain to strain, as these investigators were unable to distinguish M⁺ and M⁻ variants among either type 6 or type 49 streptococci from geographic locations other than the United Kingdom. In addition, we have observed that the degree of colony opacity can vary from serotype to serotype (unpublished data), and to make matters more complicated, the M12 strain used in this study can segregate variants capable of switching between two of three possible degrees of opacity. Early reports (17, 29, 32) which did not find a significant association between M protein and colony opacity may well have been influenced by such strain variability, or by failure to categorize colony types completely.

The relationship between opacity and the expression of M protein is clearly complex and certainly does not reflect a direct cause and effect. Although both M protein and colony opacity determinants usually switch between alternative phenotypes in phase with each other, they are genetically separable. Variants A1 and B1, despite having a stable M⁻ phenotype, continue to undergo transition between the Op⁺

and Op^- phases. It is attractive, therefore, to speculate that genes encoding these properties, although distinct, are controlled by the same genetic switch. The opacity and M protein genes could be either part of the same operon or regulated by a common *trans*-acting element. The 2-kb size of the M12 RNA transcript suggests that it is monocistronic (Robbins et al., submitted). Precedence for this type of arrangement in gram-positive pathogens is the *agr* regulon in staphylococci. The *agr* gene acts in *trans* to regulate the expression of a number of distinct genes (20). This would further explain why some strains exhibit poor correlation between colony opacity and M protein expression. Spontaneous mutations affecting one structural gene independently of the other could conceivably generate strains having one phenotype phase locked while the other continues to switch.

Using glossy colony morphology to identify M^- variants, strains CS46 and CS64, which harbor a deletion of approximately 50 base pairs, were isolated and described (26). The nearly identical location of these independent deletions suggested that deletion formation at this locus was integral to the process of phenotypic variation (26). Our more complete analysis questions this idea, as most M^- Op^- variants do not acquire a detectable deletion. Furthermore, deletions carried by phase-locked M^- variants map between 0.7 and 0.9 kb upstream of deletions in strain CS46 and CS64 (Fig. 7). Notably, all deletions map upstream of the M12 promoter (Robbins et al., submitted). We postulate that although these deletions may define a second gene required for full expression of the M12 gene, small deletions in this putative gene do not affect opacity switching and for this reason are unlikely to be a component of the phasing switch. In view of this, it remains unclear why the deletion variant A7 is phase locked in both the Op^- and M^- states. Possibly, the 3-kb deletion also affected an additional upstream gene, one associated with either colony opacity or the phase switch itself. Despite these results, it is still curious that 2 of 26 M^- variants harbored deletions. This could reflect bias in the selection procedure or mean that this segment of DNA, known to be A+T rich (Robbins et al., submitted), is a hot spot for deletion mutations.

Op^- cells able to revert to a Op^+ phenotype exhibit biphasic growth in phagocytically active blood. During the first 1.5 h of incubation, Op^- cells in Op^--Op^+ mixed cultures, such as strain A17, are phagocytized at a normal rate, but Op^- survivors of the initial 1.5-h incubation ultimately persist and multiply after 3 to 4 h of exposure to human blood (Fig. 2). This interesting observation cannot be explained by the presence of small quantities of M protein on the surface of these unstable Op^- cells because a relatively constant rate of survival was not observed. Moreover, stable Op^- cultures in which no M12 antigen or M12 mRNA was detected showed the same biphasic survival kinetics when mixed with Op^+ M^+ cells (Fig. 3). Furthermore, increasing the number of Op^+ M^+ and Op^- M^- cells in the phagocytic mixture at least 50-fold does not enhance their survival (unpublished data), indicating that the saturation of polymorphonuclear leukocytes after 2 h is unlikely to be responsible for the survival of Op^- cells. The impact of M protein and nonviable M^+ cells on the phagocytosis of M^- cells has been studied by others. Extracted M protein was found to be toxic to polymorphonuclear leukocytes (1), yet nonviable M^+ cells were not observed to alter the phagocytic killing of M^- cells (15). Our results suggest that viable Op^+ M^+ cells can inhibit phagocytosis of Op^- M^- cells but that this effect is probably dependent on a streptococcal cell product(s) which accumulates during growth. Studies are currently in progress to determine whether inhibition is the result of an extracellular or cell-bound streptococcal product and whether serum components or phagocytic cells are the target of this activity.

The creation of antigenic diversity in pilin antigens of *N. gonorrhoeae* has been linked to the pilus phase switch from P^+ to P^- (10). Although the gonococcal model involved gene rearrangements, the streptococcal phase variants described in this study did not appear to have altered M protein genes. Despite this, streptococcal M proteins are antigenically diverse, and therefore it will be important to investigate whether phase switching of M protein expression leads to altered M antigens. Thus far, employing rather coarse immunodiffusion and opsonization assays, no antigenic differences between the parental and revertant M12 antigens have been observed. Further studies are in progress to determine whether other streptococcal M types exhibit phase variation and whether this may have a role in the development of diversity in the M protein.

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