

## BACTERIOPHAGE CONTROL OF ANTIPHAGOCYTTIC DETERMINANTS IN GROUP A STREPTOCOCCI\*

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The M protein is the cell-surface antigen of group A streptococci responsible for resistance to phagocytosis by human polymorphonuclear leukocytes (1, 2). The antiphagocytic character of the M protein has long been recognized as an important virulence factor in this organism. Although the molecular basis of its activity is not entirely understood, recent reports suggest that M protein retards the activation of the alternate complement pathway and subsequent opsonization of the bacterial cell (3, 4). There are more than 85 different serotypes of group A streptococci recognizable with antisera specific for the M protein; and antibody to this protein is in part responsible for immunity to streptococcal infection. The genetic relationship of different strains and the genetic events responsible for the heterogeneity in the M antigen have not been studied.

The phenotypic variability of the M protein has been documented since Todd first isolated glossy morphological variants (5), and he and Lancefield demonstrated that they were M<sup>-</sup> (lacking the M<sup>+</sup> phenotype) and avirulent for mice (6). (Our criteria for the M<sup>+</sup> state is that the streptococcal culture resists phagocytosis and produces acid-extractable M protein.) Others have noted similar changes in M protein content during convalescence from streptococcal infection (7), and in the throats of healthy carriers (8). Previous work in our laboratory suggested a genetic basis for this instability and we proposed that extrachromosomal DNA could be responsible for the M<sup>+</sup> phenotype (9).

The literature is replete with examples of pathogenic bacteria that require bacteriophages for the maximum expression of their virulence (10), and the high incidence of lysogeny in group A streptococci is equally well documented (11). With this in mind, we began an investigation into the role of prophage in the control of M protein synthesis, limiting our initial studies to a few matched pairs (M<sup>+</sup> and M<sup>-</sup>) of laboratory strains. In this report, evidence is presented that bacteriophages are involved in the conversion of certain M<sup>-</sup> streptococci to an M<sup>+</sup> state, resistant to phagocytosis; and the implication of this information to genetic variation in group A streptococci is discussed.

### Materials and Methods

**Bacterial Strains.** Seven strains of group A streptococci were used in these studies. Strain K56 (T-12, M-12) was originally described and obtained from E. Kjemis, the Streptococcal and

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Media Departments, Statens Seruminstitut, Copenhagen, Denmark (12) and is herein referred to as strain CS24. Strain CS44, a phage A25-resistant mutant isolated from strain CS24, and strain CS64, a spontaneous, glossy M<sup>-</sup> variant of strain CS44, were originally described by Cleary and Johnson (13). Strain CS110 (T-12, M-76) also originated in the Kjems laboratory (Personal communication.) where it was referred to as strain 5641. Strain CS112 was isolated as a small colony variant of strain CS110 and purified by numerous blood agar plate (BAP)<sup>1</sup> passages; the M<sup>-</sup> character of this strain was verified by its failure to grow in human blood (lack of antiphagocytic determinants) and by its lack of extractable M antigen in unconcentrated acid extracts. A spontaneous streptomycin-resistant (str<sup>R</sup> 200 µg/ml) mutant of strain CS112 was isolated for use in these studies. Strains R72/943 (T-12, M-76), an M-76 prototype strain, and C98/105/2 (T-24, M-24), an M-24 prototype strain, were kindly supplied by Doctors W. R. Maxted and L. W. Wannamaker, respectively. The group A streptococci used in this study were classified by T agglutination (14) and the M type was determined by the capillary precipitation and immunodiffusion (15).

For typing and phagocytosis studies, bacteria were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% yeast extract (Difco Laboratories), whereas streptococci used for phage propagation were grown in No. 1 broth, as previously described (16).

*Bacteriophages.* Temperate phages were isolated and single plaques purified by common techniques. Plaque-forming units (PFU) were assayed in soft agar overlays of strain CS112 or strain CS24. Bottom agar (273) consisted of 4% proteose peptone 3, 0.2% yeast extract, 0.03 M trizma base, 0.01 M glycerolphosphate, 1.5% agar, 0.2% glucose, 0.03% CaCl<sub>2</sub>, and 68 µg/ml hyaluronidase (Sigma Chemical Co., St. Louis, Mo.) (S. Skjold. Personal communication.). Z6 top agar has been described (13).

High titer phage lysates used in the preparation of antisera were prepared as follows: a 600-ml log-phase culture of CS112 was grown in modified No. 1 broth, devoid of glucose and supplemented with 68 µg/ml hyaluronidase and 0.5% trypsin (Difco Laboratories, 1:250); after resuspension in fresh modified No. 1 broth without trypsin, the cells were infected at a multiplicity of 0.01 and incubated at 37°C for 6 h. Phage particles were concentrated by centrifugation and were resuspended in suspension medium consisting of 10 mM trizma base, 70 mM NaCl, 0.005% gelatin, and 0.1 mM MgSO<sub>4</sub> at pH 7.4.

*Production of Anti-Phage Sera.* Rabbits were subcutaneously inoculated with 2 × 10<sup>10</sup> PFU in a 1:1 mixture of phages and Freund's incomplete adjuvant (Difco Laboratories).

*Phage Curing.* Log-phase cultures of lysogens were spread on 273 plates with undiluted phage-specific antiserum and the plate was irradiated with a dose of ultraviolet light yielding 10<sup>-6</sup> colony-forming units (CFU) survival. Survivors were screened for the presence of the phage in question. Putative cured clones were purified by BAP passage, assayed for susceptibility to phages SP24 and SP44, and finally shown not to produce phage on 0.1 µg/ml mitomycin C (Sigma Chemical Co.) induction.

*Resistance to Phagocytosis.* Cultures were tested for resistance to phagocytosis by their ability to grow in human blood, employing methods previously described (9, 17). M antigen-specific opsonin was assayed by a modification of Lancefield's procedure (14) as described by Cleary et al. (18).

*Production of Anti-M Sera.* Antisera used in these studies were prepared by inoculation of rabbits with whole cell vaccines and absorbed by standard procedures (19). RS800 antiserum has been previously described (18); RS4 antiserum was made using the constructed lysogen, strain 272, as a vaccine (see Results).

*Agar Diffusion and Immunoelectrophoretic Studies.* Double-diffusion studies were done on large glass plates (10.0 × 8.0 cm) or microscope slides overlaid with double-diffusion agarose, made as previously described (18). Precipitin arcs were photographed directly, after incubation at 25°C.

Immunoelectrophoresis (IEP) studies were carried out on microscope slides as previously

<sup>1</sup> Abbreviations used in this paper: BAP, blood agar plates; CFU, colony-forming unit; IEP, immunoelectrophoresis; P1, precipitinogen 1; P2, precipitinogen 2; PFU, plaque-forming unit; SRID, single radial immunodiffusion; str<sup>R</sup>, streptomycin resistant.

described (20), with the exception that slides were incubated at 25°C after the addition of antisera.

Single radial immunodiffusion (SRID) was performed on microscope slides according to the procedure of Mancini et al. (21). These slides were also incubated at 25°C.

All immunological assays were done on acid extracts that had been concentrated by ethanol precipitation according to the method of Lancefield (22); and these concentrates were assayed for total protein by the Lowry method (23), so that equivalent amounts of acid extractable protein were always added to immunodiffusion or IEP wells.

*Trypsin Digestion.* Concentrated acid extracts were mixed with an equal volume of phosphate buffer (0.06 M phosphate, pH 7.8) containing 5.0% trypsin (Difco Laboratories, 1:250) and this mixture was incubated at 37°C for 30 min. Trypsinization was stopped by the addition of 20 mg/ml trypsin inhibitor (Sigma Chemical Co.) and another 30-min incubation at 37°C.

## Results

Strain CS112, an M<sup>-</sup>, str<sup>R</sup> derivative of strain CS110 (T-12, M-76), was chosen as the recipient because it proved sensitive to a wide range of the bacteriophages initially examined (unpublished results). Strain CS24 and its derivatives were decided upon as phage donor strains because they have different type-specific and antiphagocytic determinants (M-12) than those of the parent of the recipient strain (M-76), and because the M<sup>+</sup> phenotype of strain CS24 was known to be unstable (13).

*Bacteriophage Isolation, Comparison, and Nomenclature.* First, bacteriophages present in the phage donor and recipient strains were compared. They were isolated from the supernate of overnight cultures, and purified by three successive single-plaque isolations. Phages were named according to their origin.

Phages SP24, SP44, and SP64, originating from strain CS24 and its derivatives CS44 and CS64, formed plaques on strain CS112; and, these phages have been tentatively identified as the same by virtue of their common host range and reciprocal immunity to superinfection (Table I). This conclusion is further supported by their identical phage neutralization kinetics (using antisera directed against phage SP44) and by preliminary restriction endonuclease mapping of their respective DNAs

TABLE I  
*Sensitivity of Wild-type and Laboratory-constructed Lysogens to Bacteriophage Carried by Group A Streptococci*

Indicator lawns*	Lysis by induced cultures of strains‡			
	CS24	CS44	CS64	CS112
CS24	-	-	-	+
CS44	-	-	-	+
CS64	-	-	-	+
CS112	+	+	+	-
CS112 (SP24)	-	-	-	-
CS112 (SP44)	-	-	-	-
CS112 (SP64)	-	-	-	-

\* Strains CS24, CS44, CS64, and CS112 are wild-type strains; whereas strains CS112 (SP24), CS112 (SP44), and CS112 (SP64) are lysogens of CS112 constructed from phage that were plaque purified from the wild-type strains. Isolates were considered lysogenic if they produced phages that plaqued on the uninfected indicator strain CS112, but were resistant to superinfection by that infecting phage.

‡ (+), the formation of plaques; (-), no plaques observed.

(unpublished data). Phage SP112 produced plaques on strain CS24 and its derivatives, and did not share immunity to superinfection by those phages (Table I); thus, phage SP112 represented an unrelated phage strain harbored by the  $M^-$  indicator strain CS112.

*Construction of Lysogens.* To test the possibility that these phages could control or regulate M-protein expression, we constructed lysogens of the  $M^-$  strain CS112 by plating phage SP24 or SP44 on soft agar overlays of that strain. Lysogenic cells were picked from the turbid area within individual plaques or zones of lysis. Colonies were considered lysogenic if they produced a phage that grew on the uninfected indicator strain CS112, and were also resistant to superinfection by that infecting phage (Table I). Because all of the phage donor strains were streptomycin sensitive, an additional control was to verify that the CS112-constructed lysogens were  $str^R$ .

Our initial approach was to isolate CS112 lysogens—independent of the M phenotype—and then ask whether the M phenotype of strain CS112 had changed. Randomly picked colonies, verified to be lysogens, were then tested for their potential to resist phagocytosis. In two separate experiments, 2 out of 7 and 3 out of 10 lysogens were partially  $M^+$ , as evidenced by their survival in blood; but, they proved to be very unstable, and quickly reverted to the  $M^-$  state upon additional broth or plate passage. The precise frequency of conversion to the  $M^+$  state could not be quantitated, but one point was clear: all lysogens were not  $M^+$ .

*Selection of  $M^+$  Convertants by Rotation in Human Blood.* In an effort to stabilize the  $M^+$  phenotype,  $M^+$  lysogens were selected by repeated incubation in human blood (9). Lysogens enriched in this manner became more stably resistant to phagocytosis, whereas similar section and passaging of strain CS112 in blood invariably resulted in its eradication; any cells surviving the first exposure to blood were readily phagocytized upon reintroduction to fresh blood (Table II).

As further evidence that the  $M^-$ -recipient strain CS112 did not revert to the  $M^+$

TABLE II  
*Enrichment for  $M^+$  Cells by Incubation in Fresh Human Blood*

Strain no.	Presence of prophage	Fraction survivors*					
		Day 1 incubation		Day 2 incubation		Day 3 incubation	
		1	2	3	4	5	6
CS112	—‡	2.5	0.05	1.28	0.01	ND§	ND
272	SP24	2.65	0.80	3.56	5.39	83	1280
275	SP24	2.1	1.28	4.8	91.3	4.0	90
157	SP44	10.2	142	45.2	517	ND	ND

\* Log-phase cultures were diluted and added to freshly drawn human blood. The number of CFU added to blood was determined by viable counts, and this number was used as 1 (100% survival). The fraction of survivors equals the number of viable CFU at the indicated times divided by the number of CFU at time zero. After 3 h, survivors were added to fresh blood (1:3 dilution) and reincubated for an additional 3 h (unless otherwise indicated). After day 1, survivors were grown overnight in No. 1 broth, then transferred to Todd-Hewitt broth and yeast extract and grown to log phase before dilution into freshly drawn human blood.

‡ Strain CS112 harbors an endogenous prophage SP112, only; whereas strains 272 and 275 are independently isolated CS112 lysogens-carrying phage SP24. Strain 157 is a CS112 lysogen-carrying phage SP44.

§ ND, not done.

|| Serial transfer was made after 1 h; thus, the fraction of survivors was measured at 1 and 4 h, rather than at 3 h and 6 h.

state, independent of the bacteriophage, we did the following experiment: CS112 was serially transferred eight consecutive times into fresh human blood over a 24-h period. Despite an initial population of  $> 10^7$  CFU, no  $M^+$  survivors (resistant to phagocytosis) could be isolated from this nonlysogenic-recipient strain (data not shown).

Three independently isolated  $M^+$ -enriched lysogens, strains 157 (CS112 [SP44]) and 272 and 275 (CS112 [SP24]) were studied in more detail. If phage were required for M-antigen expression, then their loss should return the culture to the  $M^-$  state. Cured derivative strains 157c and 272c were isolated from lysogenic strains 157 and 272, respectively, after UV induction and growth in the presence of phage-specific antisera. As expected, these cured strains were sensitive to phage SP24 and SP44 and could not be induced to produce those phages by either mitomycin C or UV when lysates were assayed on the indicator strain CS112. When present, phages SP24 and SP44 are easily induced by mitomycin C. Strains 157c and 272c were purified by BAP passage, and tested for susceptibility to phagocytosis. A typical experiment is shown in Fig. 1. Phage donor strains CS24 and CS44 and strain CS112 lysogens 157, 272, and 275 multiplied in blood, clearly indicating their resistance to phagocytosis. The recipient strain CS112 and cured derivatives 157c and 272c were readily phagocytized.

*Specificity of Antiphagocytic and Precipitating Determinants of Converted  $M^+$  Lysogens.* Because these lysogens were clearly resistant to phagocytosis, it was important to determine whether they possessed M-protein determinants antigenically similar to those produced by the M-12 phage donor strain or to those produced by the parent of the  $M^-$ -recipient strain. The specificity of antiphagocytic determinants was evalu-

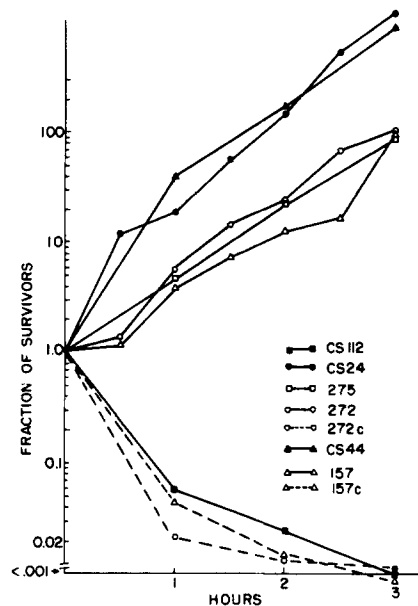


FIG. 1. Resistance to phagocytosis of phage donor and phage-recipient strains, constructed lysogens, and strains cured of their prophage. Growth in rotated blood of phage donor strains CS24 and CS44 and phage-recipient strain CS112 are compared to growth by CS112 lysogens, strains 157, 272, and 275, and phage-cured derivative strains 157c and 272c. Growth in human blood was determined as described in Table II.

ated by susceptibility to phagocytosis of cells preopsonized with type-specific opsonic antisera, because opsonic antibodies specifically prepare M<sup>+</sup> streptococci for phagocytosis. The M-76 antisera used in these experiments was RS800, made against an unrelated M-76 strain as previously described (18); whereas the M-12-specific antisera was prepared by using heat-killed cells of strain CS44 as a vaccine.

Log-phase cells were preopsonized by a 30-min incubation with preimmune or type-specific antisera, then added to freshly drawn human blood and incubated (rotating) for an additional 30 min. A comparison of viable counts taken before and after rotation in blood indicated a particular culture's susceptibility or resistance to phagocytosis. The data from these experiments demonstrated that preopsonization of strain CS110 and strain CS112 lysogens with M-76 antisera resulted in the rapid phagocytosis of those M<sup>+</sup> strains (Fig. 2). When these same cultures were preopsonized with preimmune or M-12 antisera, they remained resistant to phagocytosis. Similar preopsonization of phage donor cells, strain CS24, with M-12 antiserum resulted in their rapid phagocytosis when incubated with freshly drawn human blood. Strain CS24, however, remained resistant to phagocytosis despite preopsonization with M-76 or preimmune sera. An unrelated strain, C98/105/2 (M-24), was not phagocytized after preopsonization with either M-12 or M-76 antisera (data not shown). Therefore, we concluded that M<sup>+</sup> CS112 lysogens possessed the antiphagocytic determinants of the parental M-76 strain CS110 rather than those of the M-12 phage donor strains CS24 and CS44.

Precipitating determinants were compared by double-diffusion analysis employing concentrated acid extracts, prepared according to the method of Lancefield (22), and rabbit serum directed against one M<sup>+</sup> lysogen, strain 272. Because this antiserum (referred to as RS4) was able to precipitate acid extracts of the unrelated M-76 prototype strain R72/943 (see below), and opsonize all M-76 strains against which it was tested (unpublished data), we believed it contained antibodies directed against M-76 determinants and will refer to it as M-76 antiserum.

When the center well contained RS4, a continuous precipitin line was observed between wells containing concentrated acid extracts of strain R72/943 (an M-76 prototype strain), CS110, and the M<sup>+</sup>-converted lysogens (strains 157, 272, and 275) (Fig. 3). The common antigenic determinants shared by these strains will be referred

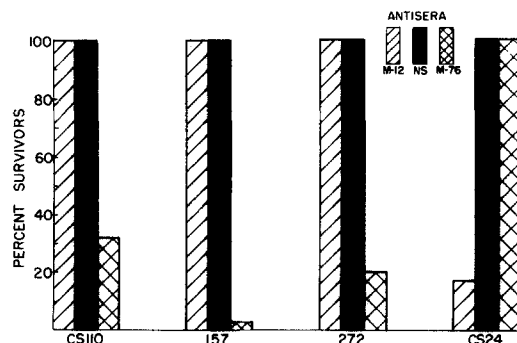


FIG. 2. Opsonization of M<sup>+</sup> lysogens by hyperimmune rabbit antisera. M<sup>+</sup> parental strains CS24 and CS110, and M<sup>+</sup> lysogens 157 and 272 were preopsonized by M-12, M-76 or normal (NS) rabbit antisera as described in Methods. The fraction of survivors equals the viable CFU after a 30-min incubation in rotated human blood divided by the CFU initially added to the blood.

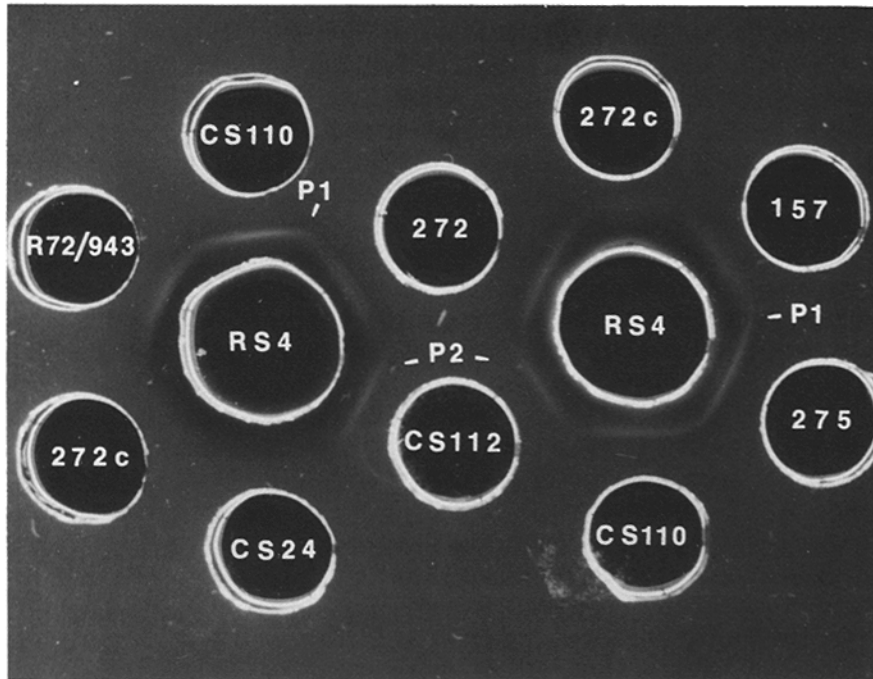


FIG. 3. Immunodiffusion analysis of  $M^+$  extracts. Center wells contain RS4 antiserum. Peripheral wells contain acid extracts, concentrated fourfold by ethanol precipitation, as described by Lancefield (22). The extract wells are labeled with the appropriate strain designations. Equivalent amounts of acid extractable protein were added to each well; protein concentration was determined by the Lowry method (23). Incubation was at 25°C for 18 h.

to as precipitinogen one (P1). P1 was not detectable in concentrated acid extracts made from strains CS112 and 272c. However, a second more peripheral precipitin line was seen in extracts made from these two strains; and, this line (P2) appeared to cross the inner common P1 line, which suggested a lack of serological relatedness between precipitinogens P2 and P1. Similarly prepared extracts of strain CS24 did not result in precipitin arcs; therefore, the RS4 vaccine strain 272, an  $M^+$  lysogen of strain CS112, did not share antigenic determinants with the M-12 phage donor, strain CS24.

Because extracts of strains CS112 and 272c contained a preponderance of P2, we could not rule out the possibility that this precipitinogen was obscuring the presence of P1 in those extracts. To test this notion, we further analyzed the acid extracts of strains CS112 and 272 by IEP on agar slides. This approach also revealed two precipitinogens (Fig. 4). The first barely moved in the electric field, migrating slowly towards the anode—a position characteristic of M proteins (18, 24). This precipitinogen was present in both 272 and CS112 preparations, although it was barely detectable in the latter. The second precipitinogen revealed by IEP was positively charged, migrating well toward the cathode. This antigen was also present in both extracts, but was more abundant in the CS112 preparation (Fig. 4).

To further distinguish these two precipitinogens and because the M protein is known to be trypsin sensitive, concentrated acid extracts from strains CS112 and 272

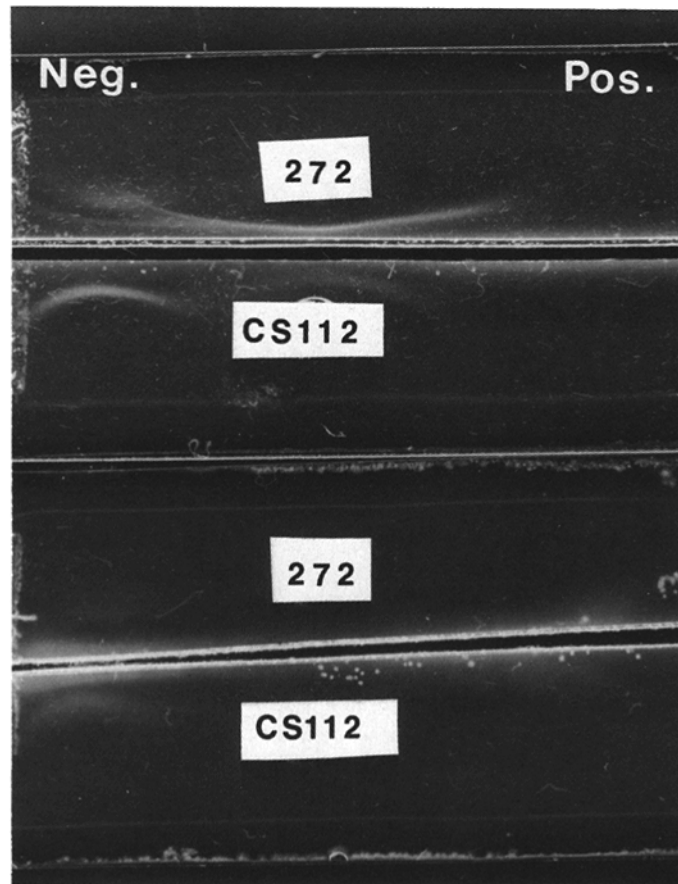


FIG. 4. Immunoelectrophoretic analysis of  $M^+$  extracts. The peripheral wells contain fourfold ethanol concentrates of acid extracts, prepared as in Fig. 3. The numbers indicate the bacterial strains examined. Equivalent amounts of extractable protein (23), were added to each well. After current was applied (9 MA/slide) for 1 h, RS4 (M-76) antisera was added to the long center trough. Slides were incubated 24 h at 25°C. Negative and positive indicate the voltage polarity. The upper slide represents untreated concentrated acid extracts, whereas the lower slide shows extracts after trypsin treatment.

were digested with trypsin. Trypsin digestion destroyed P1 (data not shown) and the less mobile antigen observed by IEP (Fig. 4). In contrast P2, presumed to correspond to the very mobile, positively charged precipitinogen was resistant to trypsin (Fig. 4). Therefore, we conclude that P1 represents M antigen and that the  $M^-$  strain CS112 may possess M antigen (but in minute amounts).

To further substantiate the finding that the  $M^-$  strain CS112 possessed some acid-extractable M antigen, M-12 and M-76 antisera were absorbed with a heat-killed preparation of strain CS112. Absorption of RS4 (M-76) antiserum removed all precipitating antibodies from that antiserum (Fig. 5). Similar absorption of M-12 antiserum did not remove M-12 precipitating antibodies; therefore, absorption of M-76 antibody from RS4 antiserum by CS112 cells was specific, and not caused by some nonspecific interaction such as Fc receptors on the surface of CS112 cells. Thus, the



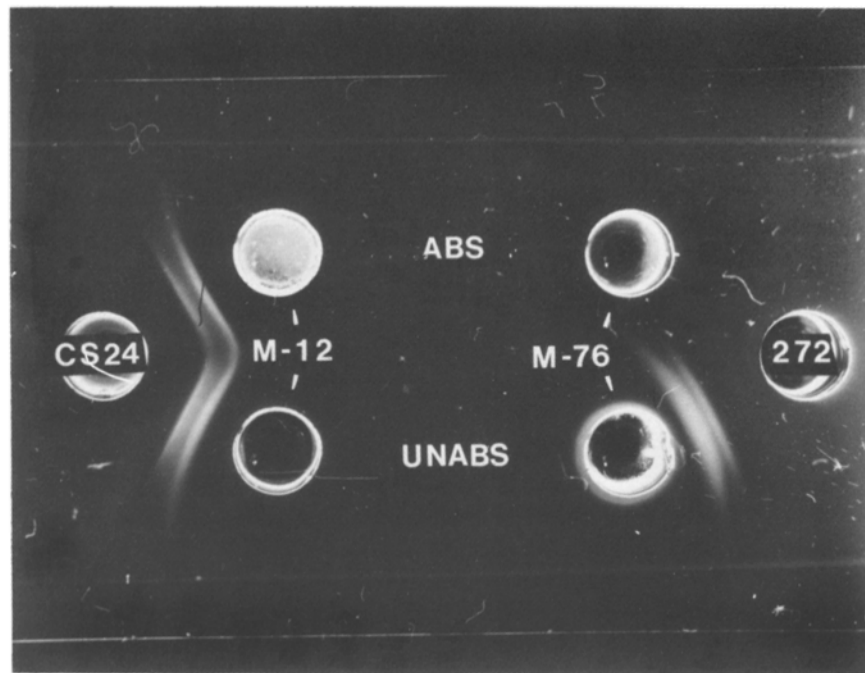


FIG. 5. Immunodiffusion analysis of absorbed hyperimmune antisera. Heat-killed cells of CS112 were prepared according to the method of Moody et al. (19), and used to absorb M-12 and M-76 (RS4) antisera as described in the Methods: UNABS indicates wells containing unabsorbed sera; ABS indicates wells containing absorbed sera. M-12 and M-76 indicate wells containing M-12 and M-76 antisera; CS24 and 272 designate the wells that contain concentrated acid extracts of strains CS24 and 272. Incubation was at 25°C for 24 h.

absorption experiments supported the contention that CS112 cells retain some M antigen, even though they are sensitive to phagocytosis.

A quantitative comparison of extractable P1 from strains CS112 and 272 was made by SRID (21). Antiserum RS4 was incorporated into immunodiffusion agar while equal amounts of acid-extractable protein from strains CS112 and 272 were added to the wells and allowed to diffuse out radially. After a 72-h incubation, a linear (dose-dependent) relationship between the amount of added antigen and the zone of precipitate in the agar slides was observed (Fig. 6). In the undiluted 272 well, there were two distinct circular precipitates indicating two distinct Ab-Ag systems. Because the outer zone disappeared after trypsinization (data not shown), we concluded that this corresponded to M antigen (P1) and that the inner band corresponded to P2 (Fig. 3). The CS112 wells similarly exhibited two distinct zones of precipitates; however, the outer, more concentrated precipitate was resistant to trypsin (data not shown), indicating that the tiny band could be M protein. Assuming that the trypsin-sensitive zone corresponded to the M antigen or P1 (Fig. 3), these assays indicated that the M<sup>+</sup> lysogen 272 possessed ~35 times more M antigen than the phage-recipient strain CS112.

#### Discussion

From the time of Pasteur, students of bacterial pathogenesis have recognized the genetic instability of virulence factors required for successful infection. Likewise, the

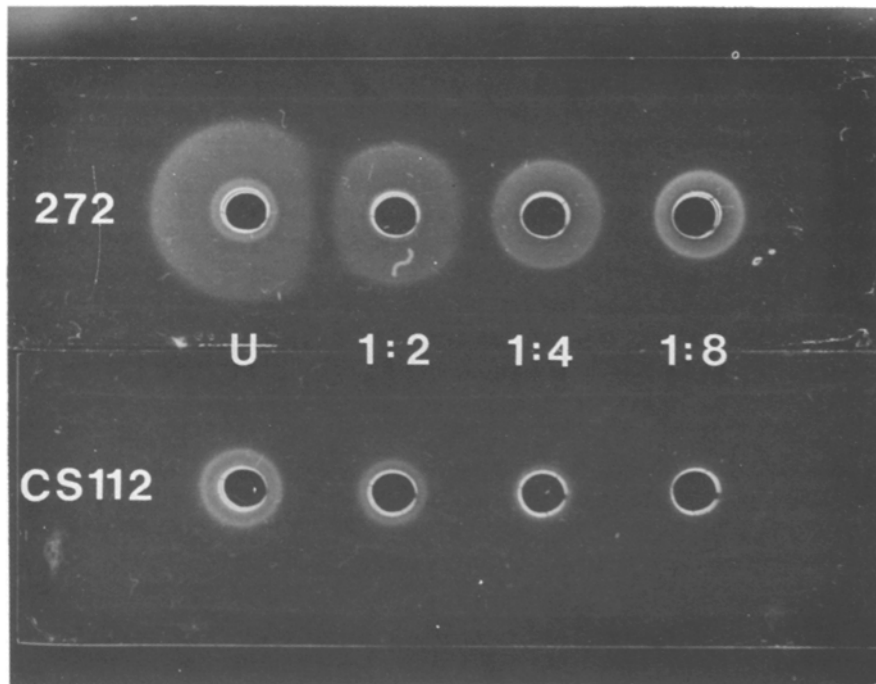


FIG. 6. Quantitation of M antigen from strains CS112 and 272 by SRID. Immunodiffusion agar contains RS4 antisera (in a final dilution of 1:8). Wells contain twofold dilutions of concentrated acid extracts of strains CS112 and 272. Equivalent amounts of acid extractable protein were added to each dilution. Slides were incubated at 25°C for 72 h.

genetic instability of the  $M^+$  phenotype of group A streptococci has been long recognized (5, 6). Investigations in our laboratory have attempted to understand the genetic basis of variation in M-protein synthesis. Initial studies showed that some  $M^-$  variants, if single colonies were carefully purified by many passages on agar plates, do not detectably revert to the  $M^+$  phenotype; and that curing agents, such as rifampicin, increase the frequency of  $M^-$  colonies in  $M^+$  cultures (9). These observations prompted us to suggest that extrachromosomal DNA is involved in the  $M^+$  phenotype. The series of experiments reported here support this possibility, and implicate temperate bacteriophages as a controlling element in M-protein synthesis.

Based on the experiments described here, we conclude that there are at least two genes involved in the synthesis of the antiphagocytic M-protein molecule: one, *mprA*, contributed by a prophage; a second, the structural gene *mprS*, contributed by the bacterium. Our evidence for phage involvement in the expression of M protein is that: (a)  $M^-$  cultures can be converted to the  $M^+$  state (resistant to phagocytosis) upon lysogenization with appropriate bacteriophages; (b) without those bacteriophages the  $M^-$ -recipient culture could not be detected to revert to the  $M^+$  state, even under our most stringent selective conditions; and (c) stable  $M^+$  lysogens cured of their bacteriophages returned to the  $M^-$  state. This phage mediated conversion is unlikely to be the result of transduction because our converting phages were always cycled through the  $M^-$  recipient, strain CS112, a number of times before conversion experiments; the M protein possessed by the  $M^+$  CS112 lysogens had the antigenic

specificity of the parental M-76 strain and not that of the M-12 phage donor strains; and the M<sup>+</sup> phenotype required that the prophage be retained by the streptococcal cells.

The structural gene, *mprS*, which determines the antigenic specificity of M protein does not reside on the prophage genome, but is either located on the streptococcal chromosome or on a yet unidentified extrachromosomal element. This conclusion is supported by the fact that M<sup>+</sup> lysogens, tested thus far, produce M antigen which is identical to that produced by the M<sup>+</sup> parent of the recipient strain, strain CS110. Antigenic determinants were compared by double-immunodiffusion analysis employing concentrated acid extracts and rabbit serum directed against one lysogen, strain 272. This serum, RS4, formed precipitates with all M-76 extracts and opsonized all M-76 strains tested, including the M-76 prototype strain. From immunodiffusion and immunoelectrophoresis analyses we were able to conclude that the parent, strain CS110, the prototype M-76 strain, and lysogens 157, 272, and 275, all have the antigen designated P1, that was presumed to be the M antigen based on its trypsin sensitivity and mobility in an electric field (18, 24). Furthermore, these strains also produced a trypsin-resistant antigen, P2, that migrated in an electric field unlike other known streptococcal products. Extracts of the M<sup>-</sup> recipient, strain CS112 and the cured strain 272c retained the trypsin-resistant antigen, P2, but had very reduced quantities of a trypsin-sensitive antigen, possibly corresponding to P1 and detectable only in concentrated extracts. Quantitation of these antigens by SRID demonstrated that extracts of the M<sup>+</sup> lysogens had ~35 times more P1 antigen than extracts from the phage recipient, strain CS112, even though both extracts contained equal amounts of total protein. Absorption studies also supported the contention that strain CS112 retains a small amount of M antigen. These cells, when incubated with antiserum RS4, removed all precipitating antibody. Because P1 was not detectable in unconcentrated acid extracts of strain CS112, and because it was barely detectable in concentrated extracts, we cannot be certain that this antigen is qualitatively the same as that isolated from other M-76 strains and M<sup>+</sup> CS112 lysogens. CS112 cells obviously retain the genetic information for M-76 antigen; therefore, it is not unreasonable to expect the synthesis of small amounts of this antigen in extracts of this strain. This result also supports the notion that the structural gene *mprS* is intact in at least some cells in the culture. Of course definitive proof that strain CS112 possesses extractable M antigen remains dependent upon the purification of the putative M antigen and subsequently showing its biochemical and immunochemical identity to P1 extracted from M<sup>+</sup> cells.

At this time, we are unable to propose a precise function for bacteriophage in the expression of the M<sup>+</sup> phenotype nor can we say with certainty that the bacteriophage genes are involved in the transition between the M<sup>+</sup> and M<sup>-</sup> states. The phage could participate directly in the biosynthesis of antiphagocytic M protein or interfere with its destruction by proteolytic enzymes. A precedent for the former is the temperate phages of *Salmonella anatum* that encode glucosyl transferases and transacetylases required for the synthesis of specific somatic antigens (25). Phage SP24, for example, could encode a cross-linking enzyme which would covalently link type-specific peptide monomers to form an antiphagocytic molecule. Fischetti et al. (26) have suggested that such an enzyme might exist in M<sup>+</sup> streptococci. Alternatively the phage could control the activity of the M-protein structural gene at the level of transcription. The

various mechanisms of transcriptional control, however, are too numerous to be discussed here.

Still unexplained, however, is the observation that many CS112 (SP24) lysogens retain their original  $M^-$  state. A trivial explanation for this is that the heterologous (M-12 and M-76) nature of the phage/host system used in these studies precludes the efficient cooperation of the *mrpA* and *mprS* genes in M-protein synthesis. Another possibility is that the "on-off" states of the  $M^+$  phenotype reflect alternate states of the prophage genome; therefore despite plaque purification, the phage SP24 lysate could consist of two subpopulations, only one of which is able to activate M-protein synthesis.

The genetic mechanism by which wild-type cells become  $M^-$  remains an open question. Random mutation in either prophage or chromosomal genes could produce the  $M^-$  state; however, we suggest that a specific genetic event is responsible. This could involve site specific inversion, deletion, or insertion of a controlling DNA segment in either chromosomal or prophage DNA. Site-specific inversion of a regulatory sequence has been shown to be responsible for phase variation in *Salmonella* (27) and in bacteriophage Mu (28); moreover, it has been recently shown that phage gene products can promote this inversion (29). The site-specific insertion of bacteriophage Mu or IS2 adjacent to or into a gene can alter that gene's expression (28); thus a specific interaction between phage SP24 and bacterial DNA could be required for the  $M^+$  phenotype. These possibilities are now under investigation in our laboratory.

### Summary

At least two genes have been shown to be required for the expression of the antiphagocytic M protein molecule in group A streptococci. Evidence for phage involvement in the expression of M protein is that: (a)  $M^-$  cultures of bacteria can be converted to the  $M^+$  state (resistant to phagocytosis) upon lysogenization with appropriate bacteriophages; (b) without those bacteriophages the  $M^-$  recipient culture could not be detected to revert to the  $M^+$  state, even under our most stringent selective conditions; and (c) stable  $M^+$  lysogens cured of their bacteriophages returned to the  $M^-$  state. Immunochemical analysis of lysogenically converted  $M^+$  strains demonstrated that they contain precipitating and antiphagocytic determinants of the parental M-76 strain (CS110) rather than M-12 determinants expressed by the phage donor strain. This information strongly suggests that the  $M^-$  strain CS112 possesses the structural gene for M protein, but that it remains predominantly unexpressed. Quantitation of the M antigen produced by these strains supports the observation that the  $M^-$  phage-recipient strain possesses a small amount of extractable M antigen and that phage activates its synthesis by some unknown mechanism. Various possibilities to account for the phage requirement in M protein synthesis and its role in the transition between  $M^+$  and  $M^-$  states are discussed.

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