CONVERSION OF AN M⁻ GROUP A STREPTOCOCCUS TO M⁺ BY TRANSFER OF A PLASMID CONTAINING AN M6 GENE

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The group A streptococcus is an important bacterial pathogen because it causes suppurative infections that may lead to the serious sequelae of glomerulonephritis or rheumatic fever. Clinical isolates of group A streptococci have M protein on their surface, and these organisms are able to resist phagocytosis in laboratory tests (1). The human host responds to streptococcal infection by elaborating antibodies to the M protein, some of which are protective against subsequent infection by streptococci of the same type (2). For these reasons, the M protein has been considered a major virulence factor for the group A streptococcus. Currently, over 70 different antigenic types of M protein have been found in nature, and this serologic diversity apparently accounts for the ability of an individual host to be infected with different strains of group A streptococci.

The M protein is found as a fibrillar molecule on the surface of the streptococcus. It is composed of coiled-coil dimers with high α-helical content (3) and is anchored to the streptococcal cell surface by its carboxy terminus (4, 5). Among streptococcal strains of different M serotypes, the molecular weight of the M protein molecule varies by over 40,000, and even within a single serologic M type, M6, the size diversity observed for M proteins is almost that great (6). However, using the cloned gene that encodes type 6 M protein (emm 6) as a DNA probe, we observed homology with strains representative of 56 different M types and three that are nontypable (7). This homology resides primarily in the carboxy-terminal part of the molecule, as shown by additional hybridization studies (8).

The DNA hybridization results revealed a group A streptococcal strain (T28/51/4) that is probably deleted for all M protein–related sequences because it does not hybridize with the emm 6 probe, although an M⁺ strain derived from the same clinical isolate does hybridize (7). Transformation of this deletion mutant allowed us to determine that the emm 6 gene we have cloned is sufficient to provide all the genetic information to convert the M⁻ deletion strain to an M6⁺ strain.
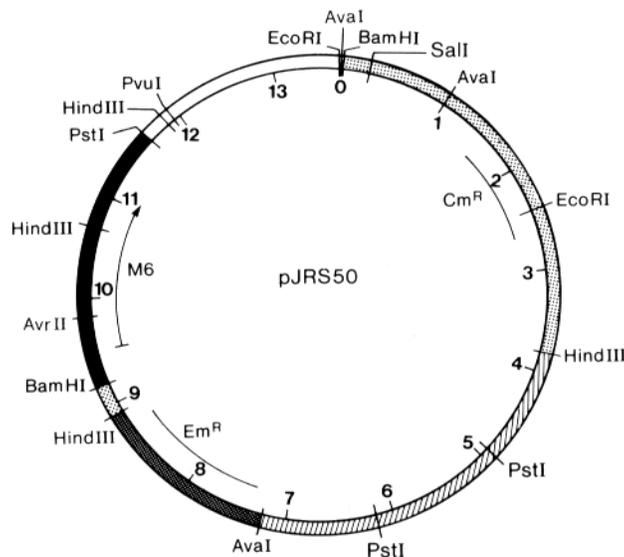
FIGURE 1.   Restriction map of pJRS50 showing the derivation of the different fragments. Both lightly stippled Bam HI–Hind III fragments (one of which carries the Cm determinant) are from pACYC184 (11), the striped Hind III–Ava I fragment is from pVA380-1 (12), the darkly stippled Ava I–Hind III fragment carries the Em determinant and is from pVA-1 (13), the black Bam HI–Pst I fragment is from *S. pyogenes* D471 and includes *emm*6 (the gene for type 6 M protein) (14), and the white Pst I–Bam HI fragment is from pUC18 (15). The numbers within the circle are kilobase coordinates.

Materials and Methods

Media and Antibiotics.   Chloramphenicol was used at 3 μg/ml for streptococci and 25 μg/ml for *Escherichia coli*, erythromycin was at 10 or 50 μg/ml for streptococci and 1,000 μg/ml for *E. coli*, and streptomycin was at 1,000 μg/ml for streptococci. Beef heart infusion broth (BHI)[1] (Difco, Detroit, MI) was used for growth of *Streptococci sanguis*, THY (Todd-Hewitt yeast extract [6]) sometimes supplemented with defibrinated sheep blood to give a final concentration of 2%, was used for *Streptococcus pyogenes*, and LB (Luria-Bertani broth) (9) was used for *E. coli*.

Streptococci Strains.   *S. pyogenes* (group A) strain D471 is a streptomycin-resistant derivative isolated in the laboratory from the M type 6 strain of The Rockefeller University (RU) collection. *S. pyogenes* strain T28/51/4, from the RU collection, is an M⁻ deletion derivative (7) obtained by laboratory passage from a clinical isolate expressing type 28 M protein. A strain resistant to 1,000 μg/ml streptomycin was selected from an overnight culture of T28/51/4 and named JRS1. *S. sanguis* V288 from Dr. F. Macrina, Dept. of Microbiology, Virginia Commonwealth University (13) is a derivative of strain Challis.

Plasmids.   pVA797 (10), from Macrina, is a conjugative plasmid in which a fragment of pVA380-1 was used to replace the erythromycin resistance (Em) determinant of the transfer-proficient plasmid pIP501. It carries the chloramphenicol resistance (Cm) determinant of pIP501.

Construction of Shuttle Vector.   pJRS50 (Fig. 1) is derived from a cointegrate of Macrina's *E. coli–Streptococcus* shuttle vector pVA838 (13) and pJRS42.50 (5), which consists of an Xba I–Pst I DNA fragment, including the *emm*6 gene from *S. pyogenes* D471 (14) cloned into pUC18 (15). We destroyed the ampicillin resistance determinant of pJRS42.50 by deleting a Pvu I fragment from the β-lactamase–encoding region of the piece of DNA

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[1] *Abbreviations used in this paper:*   BHI, beef heart infusion broth; Cm, chloramphenicol resistance determinant; Em, erythromycin resistance determinant; Sm, streptomycin resistance determinant; Tc, tetracycline resistance determinant; TH, Todd-Hewitt; THY, Todd-Hewitt yeast extract.

derived from pUC18. The resulting plasmid has two origins for replication in *E. coli* (from pUC18 and from pACYC184) and one for replication in streptococci (from pVA380-1, a cryptic plasmid from *S. ferus* [12]). It has the Cm gene of pACYC184 (11), which is not expressed in streptococci, and the Em gene from pVA-1 (13), which is expressed in both *E. coli* and streptococci. Because pJRS42.50 was inserted within the tetracycline (Tc) gene of pACYC184, the pJRS50 plasmid does not express Tc.

Transformation of S. sanguis (Strain Challis).   *S. sanguis* strain VA288 (10) was passed on BHI agar one to two times, and a single colony was used to inoculate BHI containing erythromycin. This culture was grown overnight at 37°C and diluted threefold to fresh medium containing a 10-fold dilution of heat-inactivated (56°C for 30 min) horse serum in a side-arm flask. Growth at 37°C with slow shaking was monitored in a colorimeter (Klett Manufacturing Co., Inc., New York) with a red filter. When the reading rose from 15 to 75 (which takes 2 h), 0.33 ml samples were added to the transforming DNA and incubated for 1.5 h at 37°C. Transformants were selected on THY plates containing erythromycin and chloramphenicol.

Filter Mating between S. sanguis and S. pyogenes.   The procedure of Clewell et al. (16) was followed. Recombinants were selected by plating on THY with streptomycin (to select against the donor) and erythromycin (to select for the cointegrate plasmid). The frequency was $\sim 6 \times 10^{-8}$ Em recombinants per donor CFU (based on the number of CFU on Em medium at the time of plating for recombinants). In a control mating using *S. sanguis* V288(pVA797) as the donor, there were $\sim 2 \times 10^{-5}$ Cm recombinants per donor CFU.

Colony Blot Radioimmunoassay.   This procedure was as described by Scott and Fischetti (14).

Rotation in Human Blood.   A log-phase Todd Hewitt (TH)-grown culture of *S. pyogenes* was diluted to 10–50 CFU and added to heparinized (10 U/ml) human blood. The mixture was rotated at 37°C for 3 h and survivors were plated in TH blood agar. To repeat the process, a single colony from the survivors was used as an inoculum for the next passage.

Southern Blot Analysis.   The procedures of Meinkoth and Wahl (17) was used. Washing was at conditions equivalent to 34°C below the melting temperature.

Immunofluorescence Assay.   A loopful of a log-phase culture of strain JRS2 in TH broth containing erythromycin (10 μg/ml) and strains D471 and JRS1 in TH broth were each spread on a clean glass slide and allowed to dry. The cells were fixed to the slide by immersion in methanol for 30 s and air dried. 20 μl of rabbit anti–ColiM6 IgG tagged with fluorescein isothiocyanate (18) was added to the fixed cells and the slide was incubated in a moist chamber for 30 min. The slide was washed in water and the cells were air dried. A coverslip was placed over a drop of 20% glycerol and the cells were viewed under a Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY).

Western Blot.   To quantitate the M protein, Western blots using M6 monoclonal antibodies 10A11 and 10B6 (19) were analyzed as described by Jones et al. (20) on lysin extracts.

Results

Strategy for Gene Transfer.   Because there is no system for transformation of *S. pyogenes*, we decided to use the readily transformable *S. sanguis* strain Challis as an intermediate host. A chimeric shuttle vector carrying the *emm*6 gene, pJRS50 (Fig. 1), was constructed (see Materials and Methods). Plasmid DNA extracted from *E. coli* was used to transform *S. sanguis* strain V288. Clones containing the *emm*6 gene were identified by colony blot immunoassay and the conjugative plasmid pVA797 was transferred into one of these by mating. This plasmid is composed of parts of the conjugative plasmid pIP501, which contains a Cm gene expressed in streptococci, and the small cryptic *S. ferus* plasmid pVA380-1 (21). The latter is also present in pJRS50 and provides homology between the two. Transconjugants were plated on medium containing chloram-

TABLE I

Survival in Human Blood of Strain JRS2 Compared with Its Parent JRS1

S. pyogenes strain	Inoculum (CFU)	Antiserum	Colony count after 5-h rotation
JRS1	58	—	0
	96	—	0
JRS2	32	—	424
	102	—	752
	29	Anti–Coli M6	0
	81	Anti–Coli M6	0
	29	Anti–M28	800
	81	Anti–M28	1,144

The bactericidal assay was performed as originally described by Lancefield (2), with modifications by Fischetti (22). Human blood (400 μl treated with 10 U/ml heparin) was mixed with 100 μl of a dilution of streptococci and 100 μl of either broth or antiserum. Anti–Coli M6 is hyperimmune rabbit antiserum prepared against M6 protein purified from *E. coli* (4) and anti–M28 was prepared as described by Rotta et al. (23). The mixture was rotated in capped tubes for 5 h at 37°C and a streptococcal colony count was performed by plating a 100-μl aliquot.

phenicol to select for pVA797 and erythromycin to select for pJRS50. We expected pVA797 to form a cointegrate with pJRS50, because they share a region of homology (Fig. 1) and because the two are incompatible in streptococci (21). Such a cointegrate should be transferrable by conjugation because it contains the entire pVA797 conjugative plasmid.

One *S. sanguis* Cm Em clone identified in an immunoblot assay as expressing M protein was used as a donor in filter-mating experiments with an *S. pyogenes* recipient. The recipient was a spontaneous streptomycin-resistant mutant (JRS1) derived from the *emm*-deletion strain T28/51/4 (7). An immunoblot (14) showed that all five recombinant Em Sm colonies expressed M protein.

Survival in Human Blood.   The ability of the streptococcus to survive in human blood is recognized as a means to measure the presence of M protein on the cell surface (2). To determine whether the M⁻ strain JRS1 gained the capacity to resist phagocytosis after acquiring the M6 gene, one of the five recombinant colonies was rotated in human blood. Although no net growth was observed in the blood, a number of CFU approximately equal to the number in the inoculum survived. This was in contrast to the M⁻ control (JRS1) which was killed by phagocytosis. To enrich for survivors in the recombinant strain, a phagocytosis-resistant colony was picked and the process was repeated twice. After the final rotation, a colony was selected for further study and designated JRS2. As shown in Table I, strain JRS2 survived, whereas the JRS1 parent did not.

If *emm6* encodes the M6 protein rather than activating a cryptic *emm* gene, the reconstituted JRS2 strain should have only type 6 and not type 28 M protein on its surface. Opsonization by anti-M antiserum is, for the most part, M type–specific, so we used this test to check the specificity of the protective M protein on the surface of JRS2. As expected, antiserum to purified M6 protein opsonized the JRS2 strain to allow phagocytosis, whereas hyperimmune antiserum to type 28 M protein–containing streptococci did not (Table I).
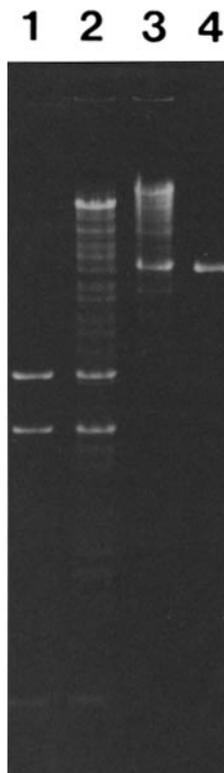
FIGURE 2.   Agarose gel of digests of pJRS50 and total cellular DNA from JRS2. Lane *1*, Pst I digest of pJRS50; lane *2*, Pst I digest of total JRS2 DNA; lane *3*, Sal I digest of total JRS2 DNA; lane *4*, Sal I digest of pJRS50.

Location of the emm6 Gene in the S. pyogenes Recombinant.   To determine the location of the *emm*6 gene in the *S. pyogenes* recipient, total DNA from JRS2 was digested with Pst I, which cuts within the pJRS42.50 part of the plasmid (see Fig. 1) and with Sal I, which linearizes pJRS50. An agarose gel revealed the presence of intact pJRS50 plasmid in strain JRS2 (Fig. 2). Hybridization of this gel with a probe consisting of almost the entire *emm*6 gene (the Nci I/Pvu II fragment; see reference 7) confirmed this (data not shown), and indicated that there was no other DNA homologous with *emm*6 in this strain. If a cointegrate with the mobilizing plasmid had persisted in the *S. pyogenes* transconjugant, or if pJRS50 had integrated into the chromosome, junction fragments between the plasmid and either pVA797 or the host should have been detectable in both of these digests. Southern blots with Bam HI and Hind III digests of total JRS2 DNA also indicated that the pJRS42.50 segment of the plasmid had not been disrupted in strain JRS2 (data not shown). Thus, the *emm*6 gene remained on pJRS50 in the transconjugant and the pJRS50 shuttle plasmid was not in the form of a cointegrate with the mobilizing plasmid.

Stability of pJRS50 in S. pyogenes JRS2.   The replicon used by pJRS50 in streptococcus is derived from a plasmid originating in *S. ferus* (pVA380-1[13]). Furthermore, there is no known stability or partition locus present in pJRS50.

TABLE II

ELISA Titers of M6 Antisera before and after Absorption with Strains D471, JRS2, and JRS1

Sera	Treatment	ELISA titer
Anti–Coli M6	Unabsorbed	25,000
	Absorbed with	
	D471	800
	JRS2	1,600
	JRS1	25,000
Anti–M6 amino-terminal	Unabsorbed	6,400
	Absorbed with	
	D471	400
	JRS2	800
	JRS1	6,400

ELISA were performed with Coli M6 protein–sensitized microtiter plates as described previously (25). The antibody titer is the reciprocal of the highest dilution yielding an OD at 405 nm of 1.0 ± 0.2. Anti–M6 amino-terminal is a hyperimmune rabbit antiserum prepared against a synthetic peptide representing the amino-terminal 20 amino acids of the native M6 protein.

Once isolated, strains containing pJRS50 have been kept under constant selection for the erythromycin resistance marker. However, because this resistance is caused by production of an altered ribosomal RNA (24) which does not turn over rapidly, many cells in the population that have lost the plasmid with the Em gene may retain the RNA that makes them resistant to erythromycin. To test for this, an overnight culture of JRS2 grown in medium containing 50 μg/ml erythromycin was plated on drug-free medium. After overnight incubation, the plates were replicated to medium containing 50 μg/ml erythromycin. In several repeated experiments, about 30% of the colonies were unable to grow on the drug medium, indicating that the plasmid is unstable in this strain.

Presence of M6 Protein on the Surface of Strain JRS2. We used two methods to test for the presence of M6 protein on the surface of JRS2. In the first test, strains JRS1 and JRS2 were checked by immunofluorescence with anti-M6 antibodies for the presence of M6 protein on the cell surface. Fluorescence was seen only with strain JRS2, and in this strain ~70% of the cells in the culture fluoresced (data not shown). This correlates with the number of cells that still retain the plasmid (see above).

In the second test, absorption of monospecific antibodies was used. In contrast to strain JRS1, strain JRS2 and M6 strain D471 absorbed M6 antibodies both from antiserum directed to the whole M6 protein and from an antiserum directed to a 20-residue synthetic peptide derived from the amino terminus of this molecule (Table II). This indicates that the M protein is present on the surface of the JRS2 strain with its amino terminus intact.

In an indirect bactericidal assay (Table III), M6 strain D471 and strain JRS2 were able to absorb opsonic antibodies from these anti-M6 antisera. Thus, the determinants necessary for opsonization are present intact on the reconstituted strain JRS2.

Western blot analysis (data not shown) of M protein extracted from strain D471 and JRS2 with phage lysin revealed the typical pattern of M protein-

TABLE III

Indirect Bactericidal Assay with M6 Strain D471 Using M6 Opsonic
Antisera Absorbed with Strains D471, JRS2, and JRS1

Antiserum	Strain with which anti-serum was absorbed	Colony count after 3-h rotation		
		Inoculum:	10	20
None	—		904	1,144
Anti–Coli M6	None		0	
	D471		736	
	JRS1		3	
	JRS2			680
Anti–M6 amino-terminal	None		19	
	D471		1,900	
	JRS1		1	
	JRS2			398

The indirect bactericidal assay using M6 strain D471 was performed as described in the legend to Table I, except that the antiserum was absorbed as indicated with different streptococcal strains and rotation was continued for only 3 h at 37°C. For absorption, overnight TH cultures of streptococci were concentrated 10-fold and heat-killed at 56°C for 45 min. Washed cells were then adjusted to an OD at 650 nm of 1.0 (using an 18-mm tube). Cells (1.5 ml) were pelleted and suspended in 300 μl of antiserum and the mixture was rotated at 4°C for 8 h. The cells were pelleted again and the serum in the supernatant was reabsorbed with a similar quantity of cells for a further 18 h at 4°C, after which the supernatant antiserum was passed through a 0.22-μm filter.

reactive bands (6) in both strains. Because solubilization of the JRS2 cell wall with lysin released the M protein, the M molecule appears to be located within the cell wall fraction. Under these conditions, the M6 molecule extracted from strain JRS2 appears slightly smaller than that extracted from strain D471. Western blots indicated that there was ~20% as much M6 protein on the surface of JRS2 as on D471.

Production of Opsonic Antibodies with Strain JRS2. To determine whether strain JRS2 elicits the production of opsonic antibodies, this strain was used to immunize two rabbits. Although some variation was observed in the response of the rabbits to this antigen, sera from both were able to opsonize the M6 strain D471 so that it was phagocytized in an indirect bactericidal assay (Table IV). Thus, we conclude that strain JRS2 has the determinants needed to elicit opsonic anti-M6 antibodies.

Discussion

We have developed a technique for introducing genes cloned in *E. coli* into the group A streptococcus, which is not transformable. The gene of interest is first cloned in *E. coli* in a shuttle vector plasmid that is capable of replicating in both streptococcus and *E. coli*. Next, an *S. sangius* Challis strain carrying both a conjugative plasmid that has homology with the shuttle vector plasmid and the shuttle vector is constructed. This *S. sanguis* strain is then mated with *S. pyogenes*, and the conjugative plasmid in the *S. sanguis* strain mobilizes the shuttle vector. The resulting *S. pyogenes* transconjugant contains two plasmids: the shuttle vector

TABLE IV

Indirect Bactericidal Assay with M6 Strain D471 Using Antisera Prepared against Strain JRS2

Antiserum	Colony count after 3-h rotation	
	Inoculum: 4	8
None	>2,000	>2,000
Anti–Coli M6	13	0
Anti-JRS2		
Rabbit 2-82	93	47
Rabbit 2-85	204	136

The procedure was as described in the legend to Table III. To immunize the rabbits, an overnight culture (500 ml) of strain JRS2 grown in TH broth containing erythromycin (10 μg/ml) was washed with PBS, pH 7.4, and heat-killed at 56°C for 30 min in 0.1 the original volume. After two additional washes, the cells were adjusted to an OD at 650 nm of 1.0. 4 ml was emulsified with an equal volume of CFA and 2.0 ml was injected subcutaneously at multiple sites in each of two rabbits. At 2-wk intervals, the rabbits were injected subcutaneously with the same quantity of cells emulsified in IFA to boost the immune response. 7–10 d after each booster dose, 20 ml of blood was removed from each rabbit.

and the conjugative plasmid from *S. sanguis.* We do not know whether a cointegrate between the two plasmids serves as an intermediate in this conjugation event. In similar conjugation experiments from *S. sanguis* to *S. faecalis,* Smith and Clewell (21) found that transconjugants did not contain a cointegrate conjugative plasmid. The streptococcal gene we have used is *emm*6, the structural gene for type 6 M protein from *S. pyogenes* strain D471. The recipient group A M⁻ strain (JRS1) has a deletion that removes all of the chromosomal region homologous to *emm*6 (7). Presumably, if there were homology between the cloned gene and the *S. pyogenes* chromosome, the plasmid could be inserted at this site by homologous recombination. Work in progress will test this hypothesis.

By use of this technique, we have added the *emm*6 gene to the M deletion strain JRS1. The introduction of the plasmid with the *emm*6 gene into JRS1 results in the presence of M protein on its surface and the ability to resist phagocytosis. We have shown that this is due to the production of M6 protein and not to reactivation of a possible cryptic defective M28 gene present in the recipient group A strain by capitalizing on the observation that opsonizing antibody for group A streptococci is M type–specific. The reconstituted M⁺ strain JRS2 absorbs M type 6–opsonizing antibody, whereas the JRS1 parent strain does not. Furthermore, antibody against JRS2 opsonizes type 6 streptococci.

Both the bactericidal test and the immunofluorescence studies indicate that the reconstituted JRS2 strain produces less M protein than D471, the strain from which *emm*6 was obtained. The pJRS50 plasmid, which carries *emm*6, is unstable in *S. pyogenes* JRS1. However, even when this instability is corrected for arithmetically, JRS2 cells that retain the pJRS50 plasmid appear to have only about one-third as much M protein per M⁺ cell as D471.

Two alternative explanations for the small amount of M protein on JRS2 are immediately apparent. First, the *emm*6 gene may be expressed less well from the

shuttle plasmid pJRS50 than from the streptococcal chromosome. pJRS50 appears to contain the natural promoter for *emm*6 (26), but, in addition, there are other promoters 5' to the streptococcal DNA that might read through *emm*6. If any of these are used preponderantly, the *emm*6 gene may not be transcribed efficiently (promoter occlusion [27]). Second, the *emm*6 promoter may be regulated by *trans*-acting factors in the streptococcal cell, and such factors may be deficient in the JRS1 host. Experiments are in progress to distinguish between these alternatives.

Summary

An M28-derived group A streptococcal strain deleted for the gene encoding M protein was converted to M⁺ by introduction of a plasmid carrying *emm*6, the structural gene for type 6 M protein from strain D471. The reconstituted M⁺ strain, JRS2, resists phagocytosis in human blood and is opsonized by anti-M6 hyperimmune serum, but not by anti-M28 serum. Immunofluorescent microscopy and ELISA demonstrate the presence of M protein on its surface. In addition, JRS2 removes opsonic antibodies from hyperimmune rabbit sera generated by immunization with purified ColiM6 protein and with a synthetic amino-terminal peptide derived from M6. Immunization of rabbits with JRS2 generates opsonic anti-M6 antibodies. These results indicate that the cloned *emm*6 gene contains the information necessary to convert a phagocytosis-sensitive streptococcus to phagocytosis resistance. Furthermore, it also contains the determinants for M type specificity and those required to elicit opsonic antibodies. It thus appears to determine all the traits associated with M protein.

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