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Virulence of Two *Streptococcus pyogenes* Strains (Types M1 and M3) Associated with Toxic-Shock-Like Syndrome Depends on an Intact *mry*-Like Gene

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The major virulence factor of *Streptococcus pyogenes*, the M protein, is positively regulated at the transcriptional level by *mry* in the M type 6 strain studied. We show here that in two *S. pyogenes* strains isolated from cases of toxic-shock-like syndrome, a type M1 strain and a type M3 strain, an *mry*-like gene is also required for resistance to phagocytosis.

The group A streptococcus, *Streptococcus pyogenes*, is a human pathogen that causes serious diseases. Primary suppurative infections, including pharyngitis and impetigo, may lead (respectively) to the more serious sequelae of rheumatic heart disease and acute glomerulonephritis. In addition, myositis and fasciitis result when this organism invades tissue. In the last 5 years, *S. pyogenes* has also been recognized as the causative agent of a severe, toxic-shock-like syndrome (TSLS) (1). TSLS is defined by isolation of *S. pyogenes* from normally sterile body sites as well as multisystem organ involvement, including hypotension, thrombocytopenia, respiratory distress, and hepatic and renal involvement (20). In addition, a generalized rash is often observed, and soft tissue necrosis may occur as well. The recent recognition of the often fatal TSLS and the observation of recurrence of rheumatic heart disease have focused attention on their causative agent, *S. pyogenes*.

The M protein present on the surface of virulent *S. pyogenes* strains protects them from phagocytosis by polymorphonuclear leukocytes (11). M proteins, which are dimeric, alpha-helical coiled-coil molecules attached to the streptococcal surface at their carboxy termini, are thus considered to be the most important virulence factors of this organism. Some anti-M protein antibodies produced after infection with *S. pyogenes* are opsonic and, therefore, protect against future infections with the same organism. This has led to the idea that a vaccine against *S. pyogenes* might be based on the M molecule. However, over 80 different serological types of M protein have been defined, and opsonic antibodies tend to be type specific. Although the carboxy-terminal region of the M molecule appears to be highly conserved (9), it is not yet clear whether antibodies to this region of the protein would be protective. Furthermore, some anti-M protein antibodies have been found to react with human tissue, especially that of the heart (4). It therefore seems appropriate to consider other approaches for prevention of streptococcal disease.

In the M6 strain JRS4, expression of M protein requires the presence of a *trans*-acting positive regulator, *mry* (3, 12). This protein is encoded immediately upstream of the structural gene for the M6 protein (*emm6*), and both genes produce monocistronic messages (3, 7). The sequence of *mry*, the structural gene for the regulatory protein, suggests that it may be the second component (response regulator) of a two-component signal transducing system (12, 18). Furthermore, like other systems regulated by signal transducers, transcription of the M protein gene is subject to environmental regulation, specifically by CO_2 concentration (2).

In a strain of type M1, a strain of type M4, and one of type M12, there are open reading frames whose sequences show more than 90% homology to the *mry* gene from the M6 strain JRS4, although the sequence information on the *mry*-like open reading frames in these three strains is not complete (6, 11a, 12, 15). In the M12 strain CS64, a small deletion in this *mry*-like open reading frame results in a loss of production of the M12 protein (17) and also of the C5a peptidase, another potential virulence factor of *S. pyogenes* (16). The peptidase is encoded downstream of *emm12* and, like the M12 protein, is produced from a monocistronic message. Thus, regulation by *mry* occurs independently for the *emm* gene and the gene encoding C5a peptidase (16). For this reason, it has been suggested that *mry* (called *virR* by Cleary's group) may be a global regulator of virulence in *S. pyogenes*.

By using polymerase chain reaction and Southern blot analysis to determine the number and homology class of the M-like genes which lie between *mry* and the gene for C5a peptidase, nine different patterns have been found among *S. pyogenes* strains of many different M types (8). Strains exhibiting four of the patterns have *mry*-related sequences, as determined by the capacity of primers derived from *mry* from the M6 strain JRS4 to amplify this DNA region (8). The other strains are postulated to have sequences partially homologous to this *mry* gene. Although the function of these *mry*-like sequences in most of these strains has not been demonstrated, the sequence information is consistent with the possibility that *mry*-like genes are widespread among divergent strains of *S. pyogenes* (8, 14) and that *mry* might be critical for expression of virulence determinants by these important pathogens. If this is confirmed, *mry* might be a potential target for antistreptococcal therapy.

The work presented here was designed to begin to determine whether *mry*-like sequences in strains other than JRS4 and CS64 are functional in regulating expression of M protein and in determining virulence. Because of the concern about the seriousness of streptococcal TSLS, we selected

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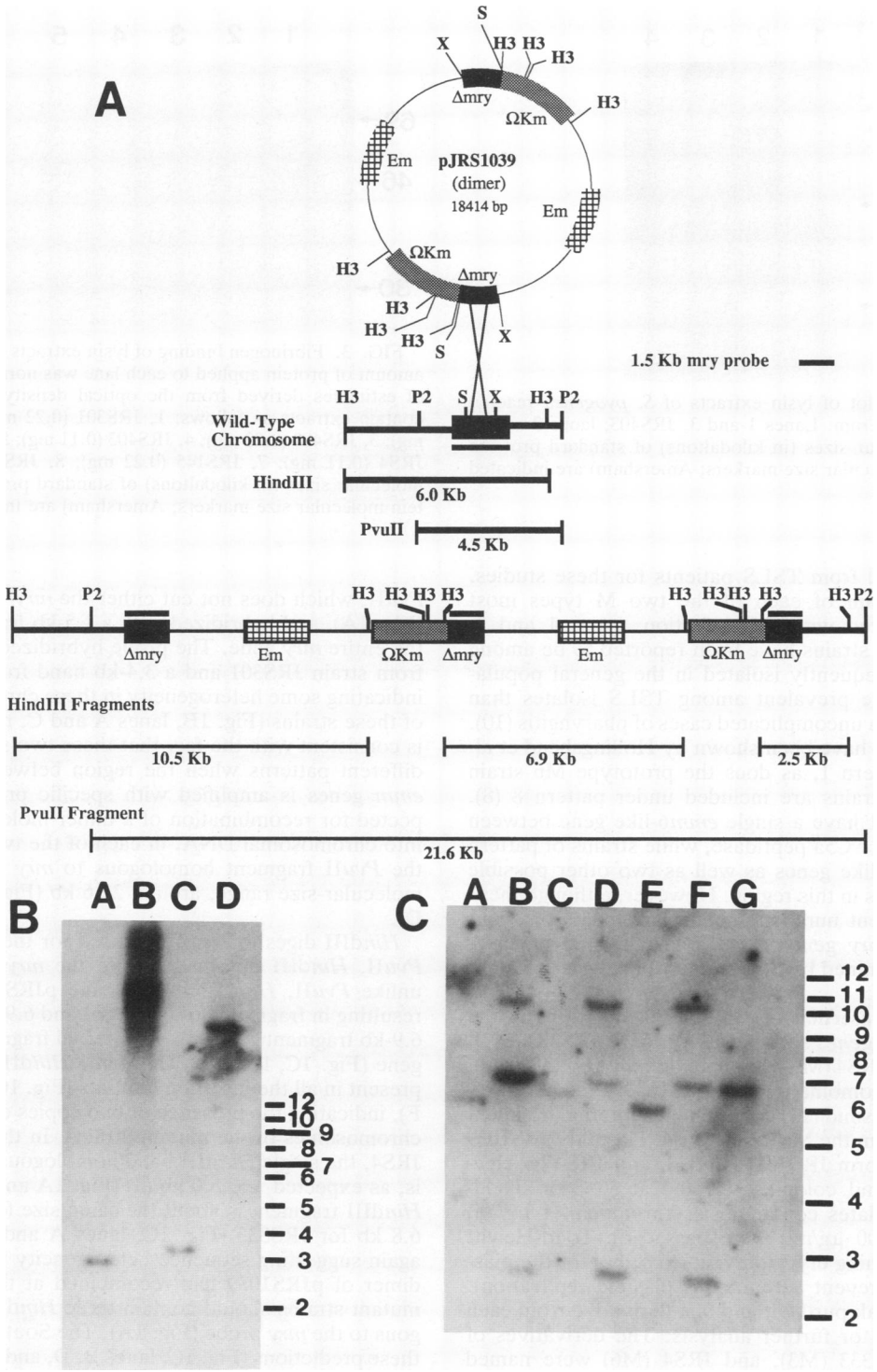

FIG. 1. Construction of pJRS1039 and recombination between pJRS1039 and the chromsome of *S. pyogenes*. (A) Plasmid pJRS1039 contains a 1-kb *Xba*I-*Sst*I segment internal to the *mry* gene (*mry*, black box), joined to the omega-Km2 element (ΩKm, stippled box) carrying signals for termination of transcription and translation (12). The vector pJRS233 (thin line) contains two replication origins, one from pSC101 and one from a temperature-sensitive replication mutant derived from pGK12, a natural gram-positive- to gram-negative-bacterium shuttle plasmid; it also contains a gene conferring resistance to erythromycin (Em, checkered box [13]). The thick lines identify additional chromosomal regions of *S. pyogenes*. Abbreviations for restriction endonucleases are as follows: H3, *Hin*dIII; P2, *Pvu*II; S, *Sst*I; X, *Xba*I. The bars underneath the chromosome of *S. pyogenes* indicate the size (in kilobases) of the fragments homologous to the *mry* probe, which is indicated by a horizontal bar above the *S. pyogenes* wild-type chromosomal D471 DNA. (B) *Pvu*II digests of chromosomal DNA of different *S. pyogenes* strains. Lanes: A, JRS301; B, JRS403; C, JRS333; D, JRS483. The molecular sizes of the standards (1-kb ladder) are indicated on the side. The probe used is shown in panel A. (C) Plasmid and chromosomal DNA digested with *Hin*dIII. Lanes: A, JRS301; B, JRS403; C, JRS333; D, JRS483; E, D471; F, JRS442; G, pJRS1039. The molecular sizes of the standards (1-kb ladder) are indicated on the side. The probe used was the same as in panel B.

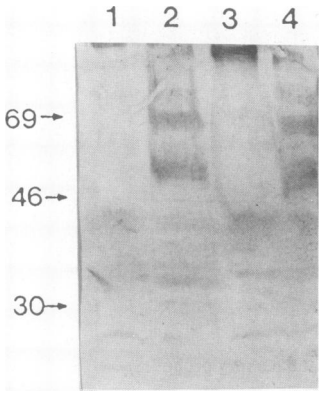

FIG. 2. Western blot of lysin extracts of *S. pyogenes* reacted with M1 typing antiserum. Lanes 1 and 3, JRS403; lanes 2 and 4, JRS301. The molecular sizes (in kilodaltons) of standard proteins (rainbow protein molecular size markers; Amersham) are indicated on the left.

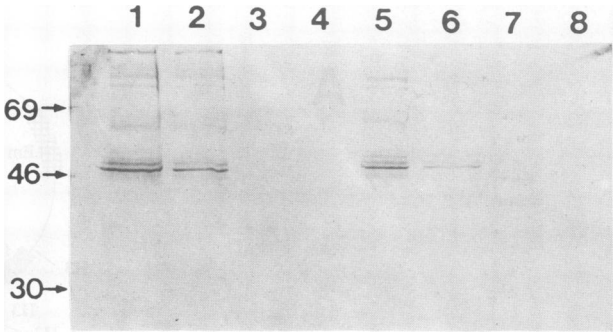

FIG. 3. Fibrinogen binding of lysin extracts of *S. pyogenes*. The amount of protein applied to each lane was normalized on the basis of estimates derived from the optical density at 280 nm. Lanes contain extracts as follows: 1, JRS301 (0.22 mg); 2, JRS301 (0.11 mg); 3, JRS403 (0.22 mg); 4, JRS403 (0.11 mg); 5, JRS4 (0.22 mg); 6, JRS4 (0.11 mg); 7, JRS145 (0.22 mg); 8, JRS145 (0.11 mg). The molecular sizes (in kilodaltons) of standard proteins (rainbow protein molecular size markers; Amersham) are indicated on the left.

two strains isolated from TSLS patients for these studies. We chose one strain of each of the two M types most commonly associated with this infection, types 1 and 3. Although M type 1 strains have been reported to be among the strains most frequently isolated in the general population, they are more prevalent among TSLS isolates than among isolates from uncomplicated cases of pharyngitis (10). Strains of M type 3 have been shown by Hollingshead et al. to conform to pattern 1, as does the prototype M6 strain JRS4, while M1 strains are included under pattern 8 (8). Strains of pattern 1 have a single *emm*6-like gene between *mry* and the gene for C5a peptidase, while strains of pattern 8 have two *emm*6-like genes as well as two other possible open reading frames in this region. However, although these strains have different numbers and arrangements of *emm*-like genes, their *mry* genes show a high degree of DNA homology as determined by Southern blot analysis (data not shown).

Construction of insertion mutants. Null mutations in the *mry* genes of *S. pyogenes* strains JRS301 (M1), JRS333 (M3), and control strain JRS4 (M6) (12) were obtained by insertion, via homologous recombination, of pJRS1039, a temperature-sensitive vector plasmid (13) containing an internal fragment of the *mry* gene from the M6 strain JRS4. Plasmid pJRS1039 was used to transform JRS301, JRS333, and JRS4 by electroporation (13), and colonies selected at 30°C on Todd-Hewitt yeast (5) plates containing erythromycin (1 μg/ml) and kanamycin (500 μg/ml) were passed on Todd-Hewitt yeast plates containing erythromycin (to select for the plasmid) at 37°C (to prevent autonomous plasmid replication). Colonies were streak purified, and one derivative from each strain was chosen for further analysis. The derivatives of JRS301 (M1), JRS333 (M3), and JRS4 (M6) were named JRS403, JRS483, and JRS442, respectively.

Confirmation of insertion mutation by Southern blot analysis. The majority of the plasmid DNA used for electroporation was in dimeric (18.4-kb) form (Fig. 1A), as indicated by agarose gel electrophoresis (data not shown). Therefore, two copies of the plasmid were expected to integrate into the *mry* gene of the recipient strains. To confirm insertion of this 18.4-kb dimeric plasmid into the *mry* gene in each strain, Southern blot analyses were performed.

Chromosomal DNA from JRS301, JRS333, JRS403, and JRS483 was digested with the restriction endonuclease *Pvu*II, which does not cut either the *mry* gene or pJRS1039 (Fig. 1A), and hybridized with a 1.5-kb fragment containing the entire *mry* gene. The probe hybridized to a 3.0-kb band from strain JRS301 and a 3.4-kb band from strain JRS333, indicating some heterogeneity in these chromosomal regions of these strains (Fig. 1B, lanes A and C, respectively). This is consistent with the fact that these two strains conform to different patterns when the region between their *mry* and *emm* genes is amplified with specific primers (8). As expected for recombination of a dimer molecule of pJRS1039 into chromosomal DNA, in each of the two mutant strains, the *Pvu*II fragment homologous to *mry* was in the large-molecular-size range, around 21.6 kb (Fig. 1B, lanes B and D).

*Hin*dIII digestion was also used for these analyses. Like *Pvu*II, *Hin*dIII does not cleave the *mry* genes; however, unlike *Pvu*II, *Hin*dIII does cleave pJRS1039 three times, resulting in fragments of 0.4, 1.86, and 6.9 kb (Fig. 1A). The 6.9-kb fragment contains the internal fragment from the *mry* gene (Fig. 1C, lane G). This same *Hin*dIII fragment is also present in all the insertion mutants (Fig. 1C, lanes B, D, and F), indicating the presence of two copies of pJRS1039 in the chromosomes of the mutant strains. In the wild-type strain JRS4, the single *Hin*dIII band homologous to the *mry* probe is, as expected, ca. 6.0 kb (3) (Fig. 1A and C, lane E). This *Hin*dIII fragment is about the same size for JRS301 and ca. 6.8 kb for JRS333 (Fig. 1C, lanes A and C, respectively), again suggesting sequence heterogeneity in this region. If a dimer of pJRS1039 had recombined at the *mry* locus, the mutant strains should contain three *Hin*dIII bands homologous to the *mry* probe (Fig. 1A). The Southern blot confirms these predictions (Fig. 1C, lanes B, D, and F), indicating that the *mry*-like genes in each strain have been interrupted by integration of the pJRS1039 plasmid dimer.

Western blots (immunoblots). The simplest test of whether the *mry* insertion mutants no longer produce M protein is Western blot analysis. Although the M3 typing antiserum was not sufficiently specific to allow identification of the M protein from this strain, the specificity of the M1 typing antiserum was adequate for this analysis. A lysin extract (5) of the M1 wild-type strain JRS301 shows the characteristic three-band pattern of an M protein (Fig. 2, lanes 2 and 4). The smallest of these bands has an apparent molecular mass

TABLE 1. Survival of *S. pyogenes* strains in human blood[a]

Strain	*mry*	M type	CFU/ml in:			
			Input	Whole blood	Plasma	Medium
JRS301	+	M1	4.9×10^2	4.55×10^5	4.04×10^5	1.98×10^4
	+	M1	1.65×10^2	1.27×10^5	1.66×10^5	1.80×10^3
	+	M1	5.4×10^2	1.60×10^5	2.03×10^5	1.04×10^5
	+	M1	3.2×10^2	3.4×10^5	2.38×10^5	5.88×10^4
JRS301/pJRS233	+	M1	2.45×10^2	1.23×10^5	1.62×10^5	5.31×10^4
JRS403	−	M1	2.45×10^2	$<6 \times 10^1$	7.32×10^4	4.92×10^4
	−	M1	2.85×10^2	$<6 \times 10^1$	2.88×10^4	6.6×10^3
	−	M1	2.33×10^2	$<6 \times 10^1$	1.90×10^5	8.70×10^3
JRS333	+	M3	9.0×10^1	1.56×10^4	8.37×10^4	7.2×10^3
	+	M3	2.0×10^1	1.1×10^5	2.08×10^5	3.42×10^4
JRS333/pJRS233	+	M3	4.0×10^1	7.2×10^4	2.62×10^5	1.86×10^4
JRS483	−	M3	2.45×10^2	$<6 \times 10^1$	3.14×10^5	6.18×10^4
	−	M3	4.35×10^2	$<6 \times 10^1$	3.19×10^5	1.72×10^5
	−	M3	2.35×10^2	$<6 \times 10^1$	1.16×10^5	9.0×10^3
JRS4	+	M6	5.6×10^2	4.02×10^4	2.94×10^4	1.68×10^4
	+	M6	3.8×10^2	1.92×10^5	3.72×10^4	3.84×10^4
JRS442	−	M6	1.2×10^3	$<6 \times 10^1$	5.28×10^4	7.02×10^4
	−	M6	4.00×10^2	$<6 \times 10^1$	4.62×10^4	1.11×10^4

[a] Assays were performed as described by Perez-Casal et al. (13).

of about 52 kDa. (The multiple bands above this band are probably composed of these M protein molecules with an associated cell wall fragment that is released during the lysin extraction procedure [5].) The extract of the M1 insertion mutant, strain JRS403 (Fig. 2, lanes 1 and 3), lacks these bands, indicating that an intact *mry* gene is required for expression of M1 in this strain.

Fibrinogen binding. To detect M protein in the absence of antiserum, we developed a blot assay based on the binding of fibrinogen to M proteins (19). Overnight cultures grown in Todd-Hewitt yeast medium plus 85 μg of hyaluronidase per ml were extracted with lysin (5), separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C in Tris-buffered saline (0.05 M Tris, 0.15 M NaCl [pH 7.5]) containing 0.25% gelatin and 0.25% Tween 20. Purified human fibrinogen (free of fibronectin) was labeled with digoxigenin by using the DIG antibody labeling kit (Boehringer Mannheim Biochemicals) at a molar ratio of digoxigenin to fibrinogen of 10:1. Bound fibrinogen was detected by using the digoxigenin detection kit (Boehringer Mannheim Biochemicals).

For these assays, an extract from the M6 strain JRS4 was included as a positive control. Fibrinogen bound to bands of the size expected (the smallest at about 53 kDa) for the M6 protein from this strain (Fig. 3, lanes 5 and 6), and no such bands were seen in the M6$^-$ deletion derivative JRS145 (Fig. 3, lanes 7 and 8) used as a negative control. The M3 strain JRS333 did not produce fibrinogen-binding protein detectable by this procedure (data not shown). However, in the extract from the M1 strain JRS301, fibrinogen bound to bands with apparent molecular masses of 52, 53, and 54 kDa (Fig. 3, lanes 1 and 2). These bands correspond in size to those detected by the anti-M1 typing serum in the Western blot. The insertion mutant JRS403 did not bind fibrinogen (Fig. 3, lanes 3 and 4), confirming the absence of M protein from this organism.

Resistance to phagocytosis. *S. pyogenes* JRS301, JRS403, JRS333, JRS483, JRS4, and JRS442 were subjected to a 3-h incubation in whole human blood or plasma at 37°C (13). The three wild-type strains, JRS301 (M1), JRS333 (M3), and JRS4 (M6), as well as strains derived from them by insertion of the vector pJRS233, multiplied in the presence either of plasma or of whole human blood (Table 1). This indicates that they resist phagocytosis by human phagocytes. In contrast, although the three mutant strains, JRS403, JRS483, and JRS442, grew in plasma, showing that there were no type-specific antibodies in the blood used, they failed to survive in whole blood.

It therefore appears that in *S. pyogenes* isolates of two different M types from TSLS cases, the *mry* gene is required for expression of the M protein. These results indicate that *mry* is an important factor in the regulation of M protein expression, possibly in all *S. pyogenes* strains, and that its production is critical for the virulence of these organisms. Efforts to develop an antistreptococcal vaccine based on the M protein have not yet been successful. Thus, it is possible that *mry* might represent an alternative target for development of antistreptococcal therapy.

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