JOURNAL OF BACTERIOLOGY, Oct. 1997, p. 6172–6180 Vol. 179, No. 19
0021-9193/97/$04.00+0
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Constitutive Expression of Fibronectin Binding in *Streptococcus pyogenes* as a Result of Anaerobic Activation of *rofA*

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Received 22 May 1997/Accepted 22 July 1997

Protein F is a fibronectin-binding surface protein of *Streptococcus pyogenes* (group A streptococcus) that mediates adherence to host cells. A gene product encoded by *rofA* activates transcription of the gene that encodes protein F (*prtF*) and was identified in a strain of *S. pyogenes* that expressed high levels of protein F under all conditions tested. Insertional inactivation of *rofA* in this strain results in a phenotype similar to that of other strains where high-level transcription of *prtF* occurs only in response to increased oxygen tension. In this study, we have compared the regulation of *prtF* and *rofA* in O_2-regulated and constitutive strains in order to gain further insight into the function of *rofA*. Comparison of the *prtF* and *rofA* transcripts by S1 nuclease and primer extension assays indicated that the same promoters for each transcript are used in both O_2-regulated and constitutive strains. However, analyses of *rofA-lacZ* reporter alleles revealed that a key difference between strains involves regulation of *rofA* itself. In O_2-regulated strains, expression of *rofA* was elevated following culture under conditions of reduced O_2 tension. However, a much more robust activation of *rofA* expression was observed when constitutive strains were grown under similar conditions. Exchange of reporter and *rofA* alleles between strains demonstrated that host genetic background, and not the sequence of the respective *rofA* allele or regulatory region, dictates the expression phenotype. Activation of *rofA* required RofA, and RofA was shown to bind specifically to DNA containing the promoters for *rofA* and *prtF*. Finally, overexpression of either allele of *rofA* caused constitutive expression of *prtF* regardless of host background. These data suggest a model where anaerobic expression of *prtF* in constitutive hosts is controlled at the level of transcription of *rofA* and implicate additional factors in this regulatory pathway.

Studies of many different bacterial pathogens have revealed that complex regulatory networks which sense environmental signals and control expression of particular sets of virulence-associated genes are essential for adaptation to different environments in the host during infection (reviewed in references 7, 24, and 26). Consequently, an understanding of how virulence factors are regulated can provide important insights into pathogenesis and the contribution of specific genes to different stages of the infection process.

An understanding of the regulation of virulence factors will be particularly valuable in the case of the gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus). This organism is an important human pathogen capable of causing a diverse set of diseases that range from relatively minor and self-limiting infections such as pharyngitis (strep throat) and impetigo to life-threatening diseases such as necrotizing fasciitis and streptococcal toxic shock-like syndrome (2). The ability of *S. pyogenes* to cause a wide spectrum of diseases in many different tissues suggests that coordinate regulation of its virulence genes plays an important role in successful adaptation to the diverse environments it encounters during infection.

Of particular interest is the regulation of the fibronectin-binding adhesin protein F (encoded by *prtF*), which has been shown to mediate binding of *S. pyogenes* to the extracellular matrix (31) and to certain populations of host cells (15, 30). Recent studies have demonstrated that *prtF* is regulated at the transcriptional level by increased oxygen levels in the atmosphere during growth (47). In addition, several lines of evidence suggest that regulation involves sensing the intracellular concentration of superoxide. These include the observation that expression of protein F was stimulated in the presence of the superoxide-generating agent methyl viologen (47) and that insertional inactivation of the gene which encodes superoxide dismutase generates a mutant hypersensitive for induction of *prtF* in response to superoxide (13).

An interesting pattern of protein F expression is exhibited by the strain from which *prtF* was originally cloned. This strain (JRS4) expresses *prtF* at constitutively high levels under all conditions examined, including the low O_2 conditions that do not stimulate expression in most other strains (47). Complementation studies with plasmid-borne alleles of *prtF* from either an O_2-regulated or the constitutive strain showed that the constitutive phenotype was dependent on a *trans*-acting factor (47). Using a m$\gamma\delta$-based insertional mutagenesis system we identified *rofA* (regulator of F), a novel transcriptional activator, as a candidate for this *trans*-acting factor (12). Disruption of *rofA* in the constitutive strain abolished the anaerobic expression of *prtF*. Unexpectedly, *prtF* expression in the *rofA* mutant was still regulated by oxygen tension (12). These studies implied that *rofA* is not involved in aerobic regulation and suggested that *rofA* alleles from O_2-regulated and constitutive strains are fundamentally different.

In order to gain further insight into the function of *rofA*, we have compared alleles from O_2-regulated and constitutive hosts. While the sequence of *rofA* from the constitutive strain was shown to differ at a number of positions from an allele from an O_2-regulated host, none of these changes could account for the difference in expression phenotype. Rather, it is shown that expression of *prtF* in both O_2-regulated and constitutive hosts is sensitive to the level of RofA and that constitutive expression results from an ability to express *rofA* at high levels under anaerobic conditions. Furthermore, the difference in expression phenotypes likely involves an additional factor involved in control of *rofA* transcription.

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Characteristic	Source or reference
E. coli			
DH5α	*recA1 endA1 hsdR17*		BRL
HB101	*recA13 proA2*		3
BL21	*ompT* [*lon*] $hsdS_B$		44
S. pyogenes			
JRS4	$prtF_{JRS4}$ $rofA_{JRS4}$	Constitutive fibronectin binding	47
HSC5	$prtF_{HSC5}$ $rofA_{HSC5}$	O_2-regulated fibronectin binding	17
SAM4	$prtF_{JRS4}$ *rofA51*::mγδ-200	*rofA* inactivated	12
HSC17	$prtF_{HSC5}$ *rofA51*::mγδ-200	*rofA* inactivatd	This work

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are described in Table 1. *Escherichia coli* DH5α was used for molecular cloning experiments, HB101 was used in the fibronectin-binding assays, and BL21 was used for protein expression. *E. coli* was cultured in Luria-Bertani broth (38) at 37°C with agitation. Culture of *E. coli* for protein expression utilized expression medium which consists of 1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.2% glucose. *S. pyogenes* was cultured in Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium) at 37°C without agitation in sealed culture tubes. Solid medium was produced by adding Bacto agar (Difco) to liquid medium at a final concentration of 1.4%. *S. pyogenes* was cultured on solid medium in ambient air (20% O_2, 0.03% CO_2) unless noted otherwise. Alternative atmospheric growth conditions were generated as described previously (4, 47). Where appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 50 μg ml^{-1} for *E. coli*; spectinomycin, 100 μg ml^{-1} for *E. coli* and 200 μg ml^{-1} for *S. pyogenes*; kanamycin, 25 μg ml^{-1} for *E. coli* and 500 μg ml^{-1} for *S. pyogenes*; and streptomycin, 1,000 μg ml^{-1} for *S. pyogenes.*

DNA manipulations. Plasmid DNA was purified by standard techniques and transformed into *E. coli* by the method of Kushner (22). *S. pyogenes* was transformed by electroporation as described previously (5, 16). Restriction endonucleases, ligases, and polymerases were used according to the recommendations of the manufacturers. Incompatible restriction fragment ends were ligated following treatment with T4 DNA polymerase to produce blunt fragment ends. For selected streptococcal transformants with modified chromosomes due to allelic replacement or insertion of an integrational plasmid, the chromosomal structure was confirmed by Southern blot analysis (43) with ^{32}P-labeled probes of the appropriate sequences.

Construction of HSC17. The inactivation of *rofA* in the O_2-regulated strain HSC5 ($rofA_{HSC5}$) was essentially identical to the construction of SAM4. In brief, a plasmid which contains the *rofA51*::mγδ-200 allele (transposon mγδ-200 inserted into the strain JRS4 *rofA* [$rofA_{JRS4}$] coding region) (12) was linearized by *Eco*RI digestion and used to transform HSC5 by electroporation. Homologous recombination resulted in kanamycin-resistant transformants in which the resident $rofA_{HSC5}$ allele was replaced by *rofA51*::mγδ-200. One of these transformants (designated HSC17) contained the expected chromosomal structure and was used in further studies.

Analysis of fibronectin-binding activity. Various *S. pyogenes* strains were cultured overnight under various conditions described in the text, and their abilities to bind ^{125}I-fibronectin were determined as described previously (15, 17) with suspensions adjusted to an optical density at 600 nm (OD_{600}) of 0.1. The data were normalized to percentages of binding activity compared to either HSC5 grown in aerobic conditions or JRS4 grown in O_2-limited conditions as noted. The data are the means and standard errors of the mean of at least two independent experiments that were performed in duplicate.

DNA sequencing and analysis. A modified T7 DNA polymerase (Sequenase 2.0; Amersham) was used to determine the nucleotide sequences of both strands of a region containing $rofA_{HSC5}$ by the dideoxy-chain termination method (37) with modifications for supercoiled plasmid templates (49). DNA sequence analysis was performed by using the Genetics Computer Group sequence analysis package (University of Wisconsin, Madison).

RNA purification. RNA from cells grown under O_2-limited conditions was isolated from *S. pyogenes* as described elsewhere (19) except that 10% (wt/vol) *N*-lauroylsarcosine was substituted for sodium dodecyl sulfate (SDS) in the lysis buffer. For aerobically grown cells, the method was modified as follows. An overnight culture of cells was diluted 1:50 in THY medium, and 100-μl aliquots were spread over the surface of each of five plates containing solid THY medium supplemented with 20 mM glycine. Following incubation at 37°C for 4 h in ambient air, bacteria were harvested with ice-cold Tris buffer (100 mM Tris [pH 6.8], 2 mM EDTA, and 0.06% [wt/vol] sodium azide). Subsequent purification was identical to that for O_2-limited cultures.

RNA analysis. Oligonucleotide primers were 5′ end labeled with ^{32}P by T4 polynucleotide kinase and hybridized to total streptococcal RNA. Primer extension analysis of each transcript was performed by reverse transcription with Superscript II RT reverse transcriptase (GIBCO Bethesda Research Laboratories [BRL], Gaithersburg, Md.) according to the conditions recommended by the manufacturer. The 5′ end of each transcript was determined by comparison to a DNA sequencing reaction generated with the same primer. Primer A (AAGTG ACAGCAAATCGCC) was used for analysis of the *prtF* RNA transcript, and primer B (TCGATTGATGATTCCAAG) was used for analysis of the *rofA* RNA transcript. S1 nuclease protection assays were performed as described elsewhere (29) with a 901-bp probe that spanned the intergenic region between *prtF* and *rofA*. The probe was generated by PCR with primer C (TCCAGGATATTCCT TACC) and primer D (GGTGGGTGGAACTATGG) and was ^{32}P labeled at either the 5′ end of primer C to detect protected fragments that corresponded to the *prtF* transcriptional start site or the 5′ end of primer D to detect the *rofA* start site. The 5′ ends of the transcripts were determined by comparing the size of the protected fragments to DNA standards of known size (100-bp ladder; BRL).

Construction of *lacZ* fusion alleles for use in *S. pyogenes*. To improve translation efficiency, a 1-kb *Bam*HI-*Cla*I fragment containing the ribosome binding site and the 5′ end of *lacZ* in the $prtF_{JRS4}$-*lacZ* reporter allele of pCMG1 (12) was exchanged for the *Bam*HI-*Cla*I fragment from pJM783 which contains a *lacZ* modified to contain the ribosome binding site of *spoVG* from *Bacillus subtilis* (10). To place the reporter allele (designated *prtF.300*) into a plasmid capable of replication in streptococci, a 6-kb *Sph*I-*Sca*I fragment from the resulting chimeric plasmid (pMGC43) was inserted between the *Sph*I and *Sca*I sites of pPTF8 (17) to form pMGC45. A $rofA_{JRS4}$-*lacZ* transcriptional fusion allele (designated *rofA.100*) was constructed by first inserting a 2.9-kb *Bam*HI-*Pst*I fragment from plasmid pPTF7::mγδ-200(50) (12) containing $rofA_{JRS4}$ between the *Bam*HI and *Pst*I sites of the *E. coli*-streptococcal shuttle vector pLZ12-Km (17). The modified *lacZ* on a 5.1-kb *Bam*HI-*Sca*I fragment derived from pMGC43 was inserted between the *Msc*I and *Bam*HI sites of the resulting plasmid (designated pPTF104) to generate pPTF125. For experiments requiring spectinomycin selection, the chimeric plasmid pPTF130 was constructed by insertion of an 8.3-kb *Sph*I-*Stu*I fragment from pPTF125 containing *rofA.100* between the *Sph*I and *Sma*I sites of the *E. coli*-streptococcal shuttle vector pAT28 (46). A transcriptional fusion allele derived from $rofA_{HSC5}$ (*rofA.200*) was generated by deletion of the 4.1-kb *Pst*I fragment from plasmid pPTF26 (47) to construct pPTF120. Subsequent insertion of the 5.1-kb *Bam*HI-*Sca*I fragment from pMGC43 containing the modified *lacZ* between the *Msc*I and *Bam*HI sites of pPTF120 generated pPTF126, which contains *rofA.200.*

Determination of β-galactosidase activity. Streptococcal cells grown overnight under the various conditions described in the text were harvested by centrifugation, washed in 1 ml of ice-cold 0.25 M Tris-HCl buffer (pH 7.5), frozen at −80°C for a minimum of 10 min, and thawed in a 37°C water bath for 5 min. Cells were diluted in Z buffer (25) to an OD_{600} of approximately 0.25. Each sample was prepared by adding 0.2 ml of the cell suspension to 0.8 ml of Z buffer, adding 50 μl of chloroform and 25 μl of 0.1% (wt/vol) SDS, and vortexing the mixture vigorously twice for 5 s on a laboratory vortex apparatus on the highest setting. The reaction mixture was allowed to equilibrate to room temperature (25°C) before 200 μl of the colorimetric substrate *o*-nitrophenyl-β-D-galactopyranoside (4 mg ml^{-1}) was added. Reactions were allowed to develop at room temperature for various times before the reaction was terminated by addition of 0.5 ml of 1 M Na_2CO_3. The mixture was centrifuged briefly to pellet the cellular debris, and the OD_{420}s of the supernatants were measured. Miller units were calculated as follows: units = $1{,}000 \times OD_{420}/(\text{time} \times \text{volume} \times OD_{600})$ (25).

Construction of a maltose-binding protein (MBP)-RofA fusion. A 1.5-kb DNA fragment that encoded $rofA_{JRS4}$ was amplified by PCR with the high-fidelity Klentaq-LA polymerase (21). The 5′ primer (CCGGATCCTTGATAGAAAAA TACTTGG) was designed to introduce a *Bam*HI site which allowed the in-frame fusion of the entire *rofA* coding sequence to *malE* encoded on pMAL-c2 (New England Biolabs). The *rofA* 3′ primer (CACTGCAGTTATGTTAATTGCTTG GTT) introduced a *Pst*I site 3′ of the coding region to facilitate insertion into pMAL-c2. Subsequent DNA sequence analysis confirmed the expected *malE*-*rofA* junction, and the resulting plasmid was designated pMBP-RofA.

A

```
JRS4 5'                           C
HSC5 5'  TATTTTGTTATTCATTTTCTCTCCTCTCAAAAACATATATGAGCTTACCACGTTATTGTCAAGAAAGACAATTTT  -143
HSC5 3'  ATAAAACAATAAGTAAAAGAGAGGAGAGTTTTTGTATATACTCGAATGGTGCAATAACAGTTCTTTCTGTTAAAA  -41
         <== Protein F *   RBS  prtF <-- start   -10                 -35

JRS4 5'
HSC5 5'  TTGATTATATTTTTCACCAAATTAATGTTTTTGAAAATGATTTTTTGAGAAAATATTGCTTTAAAGCTATTTCAA  -68
HSC5 3'  AACTAATATAAAAAGTGGTTTAATTACAAAAACTTTTACTAAAAAACTCTTTTATAACGAAATTTCGATAAAGTT  -116
                                                                          A
                                                                       B     rofA start
JRS4 5'                                  C -35                  -10
HSC5 5'  AAGGTTTCGACTTTTCACCAAAAACCATTAGATTTGATTTCTATTTTTAGCTTAGATAGAATTGAAATAGGAGAA  +8
HSC5 3'  TTCCAAAGCTGAAAAGTGGTTTTTGGTAATCTAAACTAAAGATAAAAATCGAATCTATCTTAACTTTATCCTCTT  -191

JRS4 5'              T                     G      RBS  G              * RofA ==>
HSC5 5'  TACACTTATCAAAGACTTATTTGGCTTTCTAAGTTTAGGAAGAGAGGTCCCTTTCCTTGATAGAAAAATACTTGG  +83
HSC5 3'  ATGTGAATAGTTTCTGAATAAACCGAAAGATTCAAATCCTTCTCTCCAGGGAAAGGAACTATCTTTTTATGAACC  -257
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B

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                                           helix-turn-helix
HSC5  LIEKYLESSIESKCQLIILFFKTSYLPITEVAEKTGLTFLQLNHYCEELN   50
JRS4  ----------------VV--------------------------------

HSC5  AFFPGSLSMTIQKRMISCQFTHPFKETYLYQLYASSNVLQLLAFLIKNGS  100
JRS4  ----D---------------------------------------------

HSC5  HSRPLTDFARSHFLSNSSAYRMREALIPLLRNFELKLSKNKIVGEEYRIR  150
JRS4  --------------------------------------------------

HSC5  YLIALLYSKFGIKVYDLTQQDKNTIHSFLSHSSTHLKTSPWLSESFSFYD  200
JRS4  --------------------------------------------------

HSC5  ILLALSWKRHQFSVTIPQTRIFQQLKKLFVYDSLKKSSHDIIETYCQLIF  250
JRS4  -----------------------------I-------R----------N-

HSC5  SAGDLDYLYLIYITANNSFASLQWTPEHIRQCCQLFEENDTFRLLLNPII  300
JRS4  ----------------------------------------------K---

HSC5  TLLPSLKEQKASLVKALMFFSKSFLFNLQHFIPETNLFVSPYYKGNQKLY  350
JRS4  ----N-----P---------------------------------------

HSC5  TSLKLIVEEWMAKLPGKRDLNHKHFHLFCHYIEQSLRNIQPPLVVVFVAS  400
JRS4  ------------------Y------------V--I---------------

HSC5  NFINAHLLTDSFPRYFSDKSIDFHSYYLLQDNVYQIPDLKPDLVITHSQL  450
JRS4  --------------------------------------------------

HSC5  IPFVHHELTKGIAVAEISFDESILSIQELMYQVKEEKFQADLTKQLT*     497
JRS4  -------------------------------R---------------
```

FIG. 1. Comparison of JRS4 and HSC5 alleles of *rofA* and promoter regions. (A) Comparison of the *prtF-rofA* intergenic regions from HSC5 and JRS4. The nucleotide sequences of both strands of DNA from HSC5 are shown for the region between the *prtF* and *rofA* coding regions. The nucleotide identified by S1 nuclease and primer extension assays as the start site of the *prtF* transcript is indicated by an arrow and is defined as the +1 position for the strand that encodes protein F. The start of the *rofA* transcript, which is defined as the +1 position for the strand that encodes RofA, is also indicated by an arrow. Nucleotide substitutions found in JRS4 are shown above the HSC5 sequences. The start codon of each gene is represented by a bar and an asterisk. Putative ribosome binding sites, −10 regions, and −35 promoter regions for each gene are enclosed by boxes. A TG element that is often associated with positively activated promoters is shown in boldface. The shaded bar with double arrowheads identifies a 22-bp sequence with a dyad axis of symmetry and homology to the FNR consensus binding site (AAANTTGATNNNNATCAA NTTT). The striped arrows labeled A and B identify two identical nonamer sequences that are oriented in the directions indicated. (B) Comparison of the deduced amino acid sequences of RofA from HSC5 and JRS4. The deduced amino acid sequence from the HSC5 allele of RofA is shown with the substituted amino acids in the JRS4 allele (GenBank accession number U01312) shown below. The putative helix-turn-helix motif is shown enclosed by a box. The complete nucleotide sequence of the coding region and upstream region of the HSC5 allele appears in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession no. U53825.

Expression and purification of MBP-RofA. An overnight culture of BL21(pMBP-RofA) was diluted 1:50 in expression medium supplemented with 100 μg of ampicillin ml^{-1} and was incubated at 30°C with aeration. After 3 h, expression of MBP-RofA was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.3 mM. Following an additional 2 h of incubation at 30°C, cells were harvested by centrifugation. Cell lysis and purification of the fusion protein by amylose chromatography were performed as recommended by the manufacturer (New England Biolabs). The concentration of purified MBP-RofA was determined by the bicinchoninic acid method with bovine serum albumin as a standard (42). A 500-ml culture typically yielded 12 mg of protein, of which >95% was MPB-RofA as estimated by SDS–10% polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. The fusion protein was of the expected molecular mass (99 kDa) as determined by comparison to known standards following SDS-PAGE. For certain experiments, MBP-RofA was treated with a protease (factor Xa) as recommended by the manufacturer (New England Biolabs), and the cleavage products were analyzed by SDS-PAGE.

DNA-binding studies. A 376-bp *prtF-rofA* intergenic region probe (IP) was prepared by PCR amplification with primers E (TCGATTGATGATTCCAAG) and F (AAGTGACAGCAAATCGCC) and pPTF7 (15) as the template. A 337-bp nonspecific probe corresponding to a 3′ region of the *rofA* coding sequence (RP) was amplified with the same template and primers G (CTCTATACGTC CTTAAAG) and H (CCTTTTGTAAGTTCATGG). Where indicated, these probes were labeled at their 5′ ends with ^{32}P by T4 polynucleotide kinase. Electrophoretic mobility shift analysis was performed using the following conditions. Various concentrations of purified MBP-RofA were added to a reaction containing 12 mM HEPES (pH 7.5), 12% glycerol, 0.08% bromphenol blue, 1 mM EDTA, 0.6 mM dithiothreitol, 60 mM KCl, 5 mM $MgCl_2$, and 5 fmol of labeled probe in a total volume of 25 μl. In competition experiments, various amounts of specific or nonspecific unlabeled DNA were simultaneously added to the reaction mixture. Following incubation at room temperature (25°C) for 30 min, the reaction mixtures were immediately subjected to electrophoresis through a Tris-borate-EDTA–6% polyacrylamide gel to separate free DNA from DNA-protein complexes, which were visualized by autoradiography. Quantitative measurements were performed by direct phosphorimagery of dried gels (model GS-363; Bio-Rad) with analysis using Molecular Analyst software (version 2.1; Bio-Rad).

Construction of plasmids for *rofA* expression in *S. pyogenes*. Construction of a multicopy plasmid containing $rofA_{JRS4}$ was performed as follows. A 4.2-kb *Pst*I fragment from pPTF7 (15) was inserted into the *Pst*I site of pUC19 (48) to generate pPTF101. A 3.9-kb *Sph*I fragment from pPTF101 containing the complete coding region of $rofA_{JRS4}$ was inserted into pAT28 (46) to generate pPTF128. Interruption of $rofA_{JRS4}$ was accomplished by insertion of a 2.2-kb *Sma*I fragment containing interposon ΩKm-2 (32) into the *Msc*I site in pPTF128 to generate pPTF128-Ω. A similar construct containing $rofA_{HSC5}$ (pPTF129) was produced by insertion of a 2.9-kb *Sph*I-*Xba*I fragment from pPTF23 (47) between the *Sph*I and *Xba*I sites of pAT28. Insertion of ΩKm-2 into the *Msc*I site in $rofA_{HSC5}$ in pPTF129 generated pPTF129-Ω. As a control, an integrational plasmid (pPTF127) was constructed by insertion of the entire $rofA_{JRS4}$ coding region as a 4.3-kb *Pst*I fragment from pPTF7 (15) into the *Pst*I site of integrational vector pCIV2 (29). Integration of pPTF127 into the HSC5 chromosome by a single homologous recombination event generated transformants with two functional alleles of *rofA*.

RESULTS

Comparison of *rofA* sequences in constitutive and O_2-regulated strains. The genes which encode protein F and RofA are adjacent to each other on the streptococcal chromosome but are divergently transcribed (Fig. 1A). The nucleotide sequence from this region in an O_2-regulated strain (HSC5) was determined for comparison to the homologous region in the constitutive strain (JRS4) (12). This analysis revealed several differences between the sequences. Comparison of the 266-bp intergenic regions between *prtF* and *rofA* showed five nucleotide substitutions and no deletions (Fig. 1A). Four of the five substitutions are in the 100-bp region adjacent to *rofA*. Examination of the predicted protein products from the *rofA* open reading frames from both strains ($rofA_{JRS4}$ and $rofA_{HSC5}$) identified 13 amino acid substitutions and no deletions or truncations in the primary sequence of the protein (Fig. 1B). None of the amino acid changes in the coding region lie in the putative DNA-binding helix-turn-helix motif. Based on the distribution of the amino acid substitutions, the C terminus of the protein seems less conserved than the N terminus.

A *lacZ*-based vector for analysis of streptococcal promoters using permeabilized whole cells. Further analysis of the possible impact of the nucleotide sequence differences noted above would be greatly aided by the availability of a *lacZ*-based reporter for analysis of transcription in group A streptococci. To this end, the suitability of *lacZ* modified to contain a ribosome binding site from a gram-positive bacterium (10) was evaluated by using the previously characterized *prtF* promoter region. The plasmid pMGC45, which contains the modified *lacZ* under

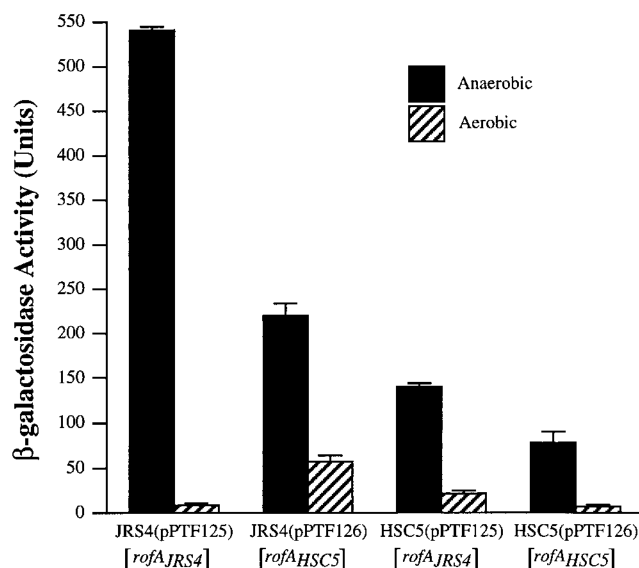

FIG. 2. Expression of *rofA* is environmentally regulated. Plasmids carrying transcriptional fusion allele *rofA.100* (pPTF125) or *rofA.200* (pPTF126) were used to transform either HSC5 or JRS4. The *rofA* alleles used to derive the fusion alleles present on the indicated plasmids are enclosed in brackets and shown below the appropriate strains. These strains were grown on THY solid medium in different atmospheres as indicated, and units of β-galactosidase activity were determined as described in Materials and Methods. Each sample was analyzed in duplicate, and the data are the means and standard errors of the mean of at least two independent experiments.

the control of the $prtF_{JRS4}$ promoter region, was introduced into O_2-regulated host HSC5, and the activity of the reporter allele (designated as *prtF.300*) was analyzed in permeablized cells following culture in different environments. β-Galactosidase activity was detected at high levels in cells following growth on solid THY medium in an ambient atmosphere (an aerobic environment; 222 ± 4.2 Miller units) and at low levels in cells grown either on solid medium in an anaerobic atmosphere (30.7 ± 1.9 Miller units) or in an O_2-limited static liquid culture (44.4 ± 4.6 Miller units). These results were consistent with previous studies of *prtF* transcription in HSC5 using a chloramphenicol acetyltransferase reporter allele (47) and paralleled *prtF*-dependent fibronectin-binding activity observed during these conditions.

Expression of *rofA* is environmentally regulated in JRS4. Plasmids containing *rofA-lacZ* fusion alleles derived from JRS4 (*rofA.100*, pPTF125) and HSC5 (*rofA.200*, pPTF126) were used to transform both HSC5 and JRS4, and the resulting strains were grown on solid medium in either aerobic or O_2-limited anaerobic atmospheres. Since *rofA* is required for the constitutive expression of *prtF* in JRS4, it was expected that *rofA* would be constitutively expressed in JRS4. Surprisingly, anaerobic growth stimulated *rofA* expression of both *rofA.100* and *rofA.200* (65- and 13-fold over the level in ambient air, respectively) (Fig. 2). While a lower level of β-galactosidase activity from each reporter allele was observed in HSC5, there was still some increase in expression in anaerobic versus aerobic growth (Fig. 2). All together, these results demonstrate that the *rofA* promoter from either an O_2-regulated or a constitutive strain is capable of high-level expression in a constitutive host background and that the *rofA* promoter is itself subject to environmental regulation.

Analysis of *prtF* and *rofA* transcripts. In order to determine if the differences in expression observed between strains were correlated with the use of different promoters, *prtF* and *rofA* transcripts were characterized by primer extension and S1 nuclease analyses. For *prtF*, primer extension revealed a major product whose 5′ end maps to a thymidine nucleotide upstream of the putative *prtF* translational start site for RNA prepared from JRS4 grown both anaerobically and aerobically and from SAM4 (an isogenic *rofA* mutant of JRS4) and HSC5 grown in aerobic conditions (Fig. 3A). While a second primer extension product was observed in the data presented for JRS4 grown aerobically, this product was not observed in additional independent reactions. In addition, S1 nuclease protection analysis revealed only a single *prtF* transcript for all strains, whose 5′ end correlated with that of the major primer extension product (data not shown). Similarly, primer extension analysis revealed a single *rofA* transcript in all strains, whose 5′ end corresponded to a thymidine located 64 nucleotides upstream of the putative *rofA* translational start site (Fig. 3B). In agreement with previous data (11) and the analysis of reporter alleles, it was consistently more difficult to obtain a *rofA* primer extension product from SAM4 and HSC5 following aerobic growth. The locations of these transcript ends were consistent with the results of S1 nuclease protection analysis (Fig. 3C and data not shown) with the exception that no protected fragment was obtained with samples prepared from *rofA* mutant SAM4 grown under anaerobic conditions (Fig. 3C). The locations of the transcriptional start sites and putative promoter −10 and −35 regions are shown in Fig. 1B and are considered in greater detail in the Discussion section. However, taken together these results show that both O_2-regulated and constitutive hosts use the same promoters for expression of *prtF* and *rofA*. In addition, the analysis of *rofA* mutant SAM4 indicates that the start site of *prtF* transcription is not altered by the presence or absence of RofA.

High-level anaerobic expression of *rofA* requires RofA. The failure to detect an S1 nuclease protection product for *rofA* in SAM4 suggested that like the streptococcal activator protein Mga (previously Mry or VirR) (29), RofA may participate in regulation of its own gene. To test this, the *rofA.100* reporter allele contained on pPTF130 was introduced into *rofA* mutant SAM4 and into JRS4 and HSC5 for comparison. Similar to previous results, a high level of β-galactosidase activity was only detected in JRS4 following anaerobic growth (Fig. 4), although a modest increase (threefold relative to aerobic growth) was observed under microaerobic conditions (5% O_2, 10% CO_2) (Fig. 4). Significantly, inactivation of *rofA* prevented high-level anaerobic expression of the *rofA* promoter [see SAM4(pPTF130), Fig. 4].

RofA binds the *rofA-prtF* intergenic region. Since RofA is involved in activation of its own expression as well as that of *prtF*, and since it contains a putative DNA-binding domain (12) (Fig. 1B), it was of interest to determine if RofA directly interacted with the DNA region containing the *prtF* and *rofA* promoters. To accomplish this, a chimeric protein containing the entire *rofA* coding sequence joined to an MBP was constructed (MBP-RofA) (see Materials and Methods). Treatment of MBP-RofA with factor Xa to cleave the chimeric protein at the single factor Xa site at the junction between MBP and RofA generated the expected 43-kDa (MBP) (8) and 56-kDa (RofA) (12) fragments (data not shown). However, similar to an MBP-Mga fusion protein (23), cleavage proved to be inefficient, and subsequent experiments utilized the untreated chimeric protein. Binding of MBP-RofA to a DNA probe containing the *prtF-rofA* intergenic region (IP) was demonstrated by the appearance of a band with reduced mobility compared to that of free IP after gel electrophoresis (Fig. 5, lanes 2 to 4, IP + MBP-RofA). The gel retardation effect was

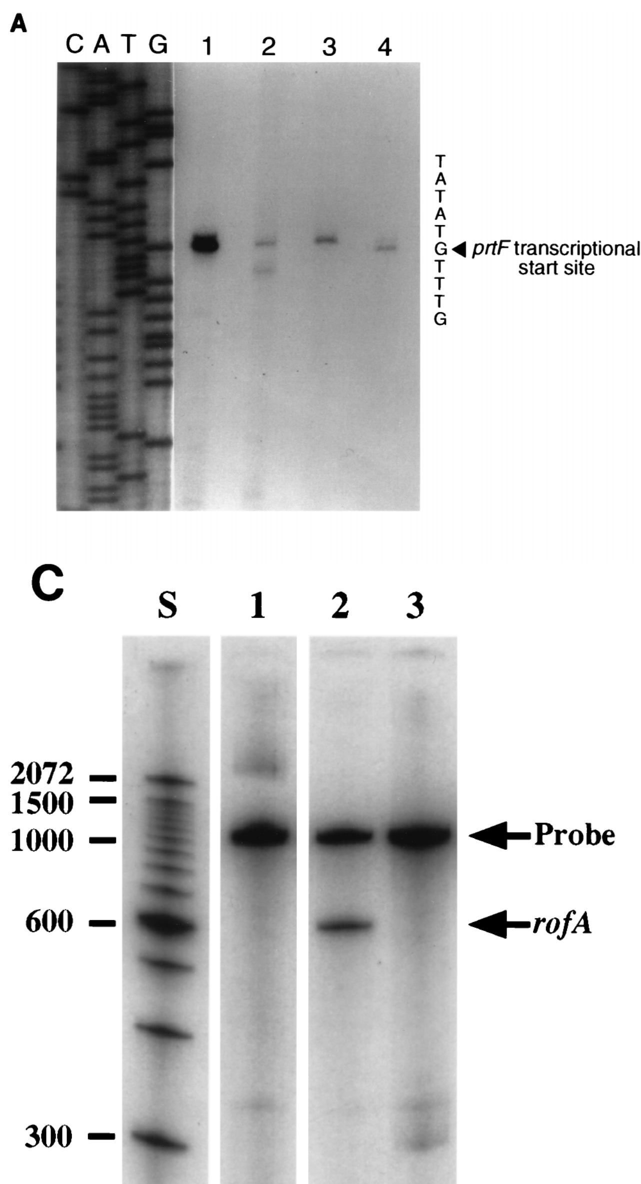

FIG. 3. Analysis of *prtF* and *rofA* transcripts. (A) Primer extension analysis of *prtF* transcripts generated from total RNA isolated from JRS4 grown in O_2-limited conditions (lane 1) and from JRS4, SAM4, and HSC5 grown in aerobic conditions (lanes 2 through 4, respectively). Total RNA was hybridized with primer A (see Materials and Methods) labeled at the 5′ end with ^{32}P, and an extension product was generated with reverse transcriptase. Primer A was also used to generate the dideoxynucleotide-terminated sequencing reactions, labeled C, A, T, and G, which correspond to the DNA sequence of the strand complementary to the transcript. DNA from JRS4 served as the template for the sequencing reactions. The arrowhead indicates the 5′ end of the *prtF* transcript. (B) Primer extension analysis of *rofA* transcripts generated from the same RNA samples described above for panel A. All experimental conditions were identical to those described above except that reactions used primer B (see Materials and Methods), which was also used to generate the dideoxynucleotide sequence reactions in the lanes labeled C, A, T, and G, which correspond to the DNA sequence of the strand complementary to the transcript. DNA from JRS4 served as the template for the sequencing reactions. The arrowhead indicates the 5′ end of the *rofA* transcript. (C) S1 nuclease protection analysis of *rofA* transcripts from JRS4 (lane 2) or SAM4 (lane 3) using RNA prepared from cells grown under O_2-limited conditions. Yeast tRNA (lane 1) is included as a control, and lane S contains labeled DNA standards (100-bp ladder; BRL). The sizes of selected standard molecules (in base pairs) are indicated on the left. The mobilities of the undigested probe and the product protected from digestion by the *rofA* transcript are indicated on the right.

dose dependent with 11 pmol of MBP-RofA completely shifting the probe into the complexed state, while 0.11 pmol of protein left most of the probe in the unbound state (Fig. 5, compare lanes 2 and 5). Furthermore, with higher concentrations of MBP-RofA significant amounts of a high-molecular-weight product which did not enter into the gel and a considerably smeared band migrating at the level of the IP–MBP-RofA (IP + MBP-RofA) complex and above (Fig. 5, lanes 2 and 3) were consistently observed. It is unclear if this behavior resulted from the formation of higher-ordered protein-DNA complexes or was the result of protein aggregation. The approximate apparent disassociation constant (K_d^{app}), defined as converting 50% of the IP into the complex, was 100 nM. Specific binding was demonstrated by the observation that the addition of 100- and 10-fold excess unlabeled specific probe competed for complex formation with labeled specific probe (Fig. 5, lanes 6 and 7). Addition of 200- and 50-fold excess of a probe derived from the 3′ end of *rofA* (RP) was unable to compete for binding with the specific probe, although it did cause the IP + MBP-RofA complex to smear (Fig. 5, lanes 8 and 9). The MBP moiety did not contribute to binding as shown by the failure of an alternative MBP fusion to an unrelated *E. coli* protein (18) to form a complex with IP (Fig. 5, lane 10). As a final test of specificity, complex formation with IP was only observed when a reaction mixture containing equal quantities of labeled IP and RP was incubated with MBP-RofA (Fig. 5, lane 13).

Overexpression of *rofA* confers the constitutive phenotype. As predicted by the ability of *rofA* mutant SAM4 to activate *prtF* expression under aerobic conditions, inactivation of *rofA* in O_2-regulated strain HSC5 did not effect aerobic stimulation of fibronectin binding (Fig. 6, compare HSC5 and HSC17). However, *rofA* is capable of activating *prtF*, and analyses of promoter activities suggested that activation was associated with high-level expression of *rofA* itself. To expand this analysis, the effect of expression of *rofA* from a multicopy plasmid was investigated. Either allele of *rofA* was capable of efficient complementation of the *rofA* mutation in SAM4 when present on a high-copy-number plasmid (pPTF128 contains $rofA_{JRS4}$, and pPTF129 contains $rofA_{HSC5}$) (Fig. 6). In addition, $rofA_{HSC5}$ supported expression of *prtF* in this host even under O_2-limited

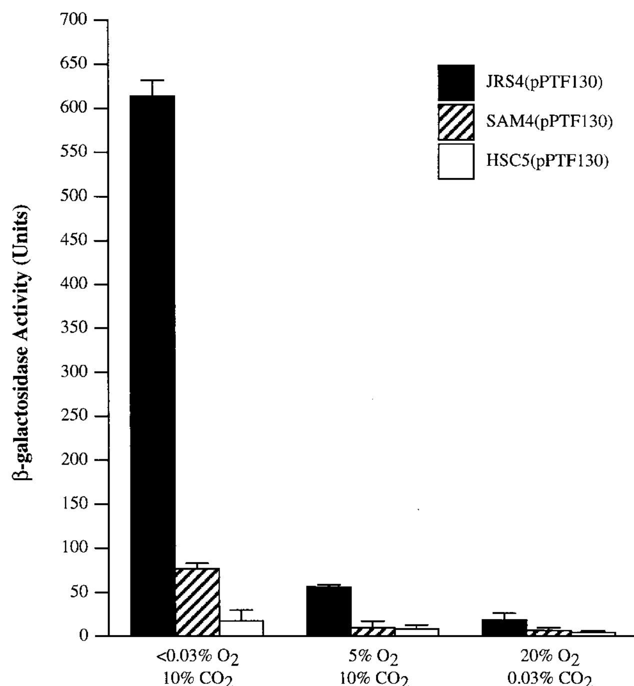

FIG. 4. The expression of *rofA* is positively autoregulated. Plasmid pPTF130, which contains the transcriptional fusion allele *rofA.100* (derived from $rofA_{JRS4}$), was used to transform JRS4, SAM4, and HSC5. These strains were grown on THY solid medium in the different atmospheres indicated, and the β-galactosidase activities were determined as described in Materials and Methods. Each sample was analyzed in duplicate, and the data are the means and standard errors of the mean of at least two independent experiments.

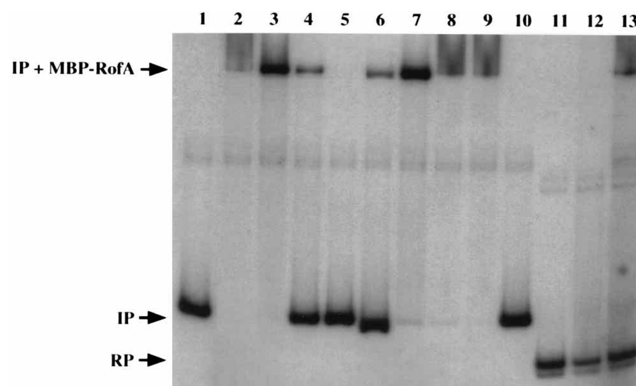

FIG. 5. Electrophoretic mobility shift assay of DNA containing the *rofA* and *prtF* promoters by MBP-RofA. A labeled DNA probe containing the *rofA* and *prtF* promoters (IP, 5 fmol) was incubated with no protein (lane 1) or with 11, 5.5, 1.1, and 0.11 pmol of MBP-RofA (lanes 2 to 5, respectively), and the formation of DNA-protein complexes was visualized by autoradiography following electrophoresis through a Tris-borate-EDTA–6% polyacrylamide gel. Competition for binding 5 fmol of labeled IP to 11 pmol of MBP-RofA was performed by the addition of 500 and 50 fmol of unlabeled IP (lanes 6 and 7, respectively) or 1,000 and 200 fmol of a DNA probe derived from the 3′ end of *rofA* (RP; lanes 8 and 9, respectively). An unrelated MBP fusion protein (1.8 μg) (18) was incubated with 5 fmol of labeled IP (lane 10). Labeled RP (5 fmol) was incubated with no protein or with 11 pmol of MBP-RofA (lanes 11 and 12, respectively). A reaction mixture containing both labeled IP and RP (5 fmol each) was incubated with 11 pmol of MBP-RofA (lane 13). The relative mobilities of free RP, free IP, and the IP + MBP-RofA complex are indicated on the left.

conditions (Fig. 6, pPTF129). Significantly, introduction of either plasmid-encoded allele converted O_2-regulated strain HSC5 to a constitutive phenotype where high levels of fibronectin binding were observed under both aerobic and O_2-limited conditions (Fig. 6). Insertional activation of the plasmid-encoded *rofA* alleles prevented activation (Fig. 6, pPTF128-Ω, pPTF129-Ω), suggesting that the effect was mediated by the high copy number of *rofA* and not due to titration of a soluble factor. In addition, integration of $rofA_{JRS4}$ into the *rofA* locus of HSC5 to produce a chromosome with two functional tandem copies of *rofA* was also not sufficient for conversion to the constitutive phenotype (data not shown), providing further evidence that this effect was dependent on *rofA* at high copy number. These data demonstrate that even in an O_2-regulated host background, *prtF* can be activated in *trans* by elevated levels of either form of RofA.

DISCUSSION

In this study, we have compared *rofA* alleles and the regulation of *rofA* and *prtF* in a strain that constitutively expresses *prtF* (JRS4) and a strain that regulates *prtF* in response to O_2 levels in the environment (HSC5). These data are consistent with a model in which transcription of *prtF* is sensitive to the level of RofA. In addition, RofA likely acts as a transcriptional activator that operates through binding to DNA in the vicinity of the *prtF* and *rofA* promoters. However, it should be noted that the data do not exclude the possibility that the effects of RofA are mediated posttranscriptionally. Furthermore, RofA participates in regulation of its own expression and can itself be subject to environmental regulation. The latter point apparently explains the constitutive expression phenotype of JRS4 which, unlike the O_2-regulated strain, upregulates transcription of *rofA* under anaerobic conditions. These results also demonstrate that *rofA* has the capacity to participate in the activation of *prtF* in other strains, which implies that other environmental conditions may lead to *rofA* activation in these strains. Finally, the fact that host background had more influence on the expression phenotype than any specific *rofA* allele suggests that additional factors participate in the expression and/or activation of *rofA*.

An unexpected observation was that the constitutive expression phenotype of JRS4 is not the result of constitutive expression of *rofA*. Rather, constant high-level activation of *prtF* occurs through overlapping pathways, a *rofA*-dependent pathway during anaerobic growth and a *rofA*-independent pathway during aerobic growth. The independence of these two pathways is supported by the observation that *rofA* had a negligible role in *prtF* activation during aerobic growth in all strains examined. However, the observation that *rofA* has the capacity to activate *prtF* in all strains suggests that it may function as part of a signaling pathway that responds to an unknown environmental cue. It could be advantageous to have a mechanism for expression of fibronectin-binding activity under conditions that are not necessarily those of aerobic environments. For example, protein F in combination with fibronectin allows the bacterium to bind to collagen-containing structures in the more O_2-limited environment of deep tissues like the dermis (30).

Based on the precedent that mutations in other activator proteins can result in a constitutive phenotype (1, 36), it was anticipated that one or more of the sequence differences noted between the *rofA* alleles would be the basis of constitutive expression of *prtF*. However, this is not likely to be the case since it was shown that both alleles of *rofA* could efficiently activate *prtF* in any host when overexpressed and that integration of $rofA_{JRS4}$ into the HSC5 chromosome did not result in

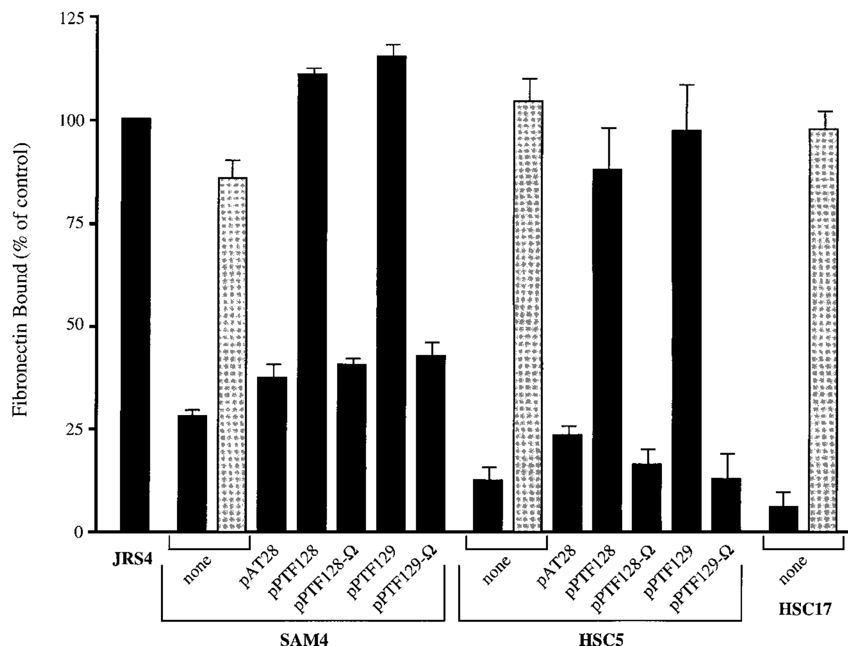

FIG. 6. Overexpression of *rofA* with multicopy plasmids. The abilities of the indicated strains to bind ^{125}I-fibronectin following growth in O_2-limited conditions (liquid THY medium in sealed tubes without agitation [■]) were determined as described in Materials and Methods. Binding was quantitated relative to the binding of JRS4 grown under O_2-limited conditions. The plasmids pPTF128 and pPTF129 contain $rofA_{JRS4}$ and $rofA_{HSC5}$, respectively. In plasmids pPTF128-Ω and pPTF129-Ω, *rofA* has been interrupted by ΩKm-2. Plasmid pAT28 is the vector control, and "none" indicates that a plasmid has not been introduced into the indicated host. HSC17 (*rofA51*::mγδ-200) is derived from HSC5 by insertional inactivation of *rofA*. For comparison, the binding activities of SAM4, HSC5, and HSC17, each with no introduced plasmid, were determined following growth under aerobic conditions (solid medium in ambient air [▭]). Data are the means and standard errors of the mean of a minimum of two independent experiments.

constitutive expression. Since it appears that the expression phenotype predominately reflects host background rather than any specific *rofA* allele, an alternative explanation may be that the difference resides in a gene which encodes a product that controls expression and/or activation of *rofA*. The origin and significance of this phenotypic difference is not clear; however, previous introduction of $prtF_{JRS4}$ into an M1 strain of *S. pyogenes* resulted in constitutive high-level expression of fibronectin binding under anaerobic conditions (17). While many M1 strains lack a protein F that contains fibronectin-binding domains (11, 28), they do possess *rofA* (11, 45). The results with this M1 strain suggest that heterogeneity in activation of *rofA* may not be restricted only to the strain analyzed in the present investigation.

Examination of the region upstream of the *prtF* transcription start site revealed sequences at the −10 (TAAGCT) and −35 (TTGTCT) positions (Fig. 1A) that resemble the canonical σ^{70} −10 (TATAAT) and −35 (TTGACA) boxes for gram-positive organisms (14, 27). Not unexpected for a promoter requiring activation, there is some divergence from the canonical sequences; however, highly conserved residues are present in the −10 region, including an adenosine at position 2, a thymidine at position 6 (35), and a TG sequence upstream of the −10 region (Fig. 1A) that is important in several other positively activated promoters (20, 34). Based on the location of this promoter, it is likely that the initiation codon is located at a position 19 nucleotides from the transcription start site (Fig. 1A) rather than at the position predicted previously (39). This new position also predicts a highly favorable ribosome binding site (41) centered 7 nucleotides upstream of the translation start site. Although the significance of the relatively short 5′ untranslated region in this transcript is unclear, other positively activated genes from *S. pyogenes* have similar transcripts including the Mga-activated *scpA6*, which has an even shorter 16-nucleotide 5′ untranslated region (33).

Based on analysis of reporter alleles, the HSC5 *rofA* promoter did show approximately twofold lower activity than the JRS4 *rofA* promoter, indicating that the HSC5 allele has a slightly weaker promoter. Examination of the putative *rofA* promoter sequences revealed that they correspond closely with the canonical sequences with a −35 region (TTGATT) that matches at four of six positions and a −10 region (TAGAAT) that matches at five of six positions (Fig. 1A). Comparison of the JRS4 and HSC5 sequences in this region show a C to T transitional mutation that lies immediately upstream of the −35 box which could presumably affect the activity of this promoter in HSC5 (Fig. 1A). However, the HSC5 promoter was still strongly upregulated by anaerobic conditions in the JRS4 background, suggesting that this difference was not sufficient to account for the constitutive phenotype.

Several sequence elements were identified as candidates for participation in regulatory control, including two identical TTGAAATAG nonamer motifs in opposite orientation (Fig. 1A). One of these repeats overlaps with the −10 region of the *rofA* promoter, while the second overlaps with a 22-nucleotide palindromic sequence that matches the consensus binding sequence for the *E. coli* global regulator FNR (9) at 17 of 22 positions (Fig. 1A). FNR regulates numerous genes in response to O_2 tension in *E. coli*, and a homolog of FNR has recently been cloned from the gram-positive bacterium *B. subtilis* (6). Thus, it is interesting to speculate that these sequences and a streptococcal FNR homolog are involved along with RofA in regulation of these promoters.

While RofA lacks the critical residues which are characteristic of members of the FNR family of regulators (40), it does have a putative DNA-binding domain (12), and in this study it

was shown to bind specifically to a region which includes the *prtF* and *rofA* promoters. RofA does possess limited homology (12) to the virulence activator Mga, which also participates in its own regulation and has been shown to bind to a characteristic nucleotide sequence in its target promoters (23). However, the regions of RofA-Mga homology do not include the regions of Mga which possess similarity to the receiver domain of a two-component regulatory system. Thus, additional studies, including further analysis of the interaction of RofA with DNA, identification of other genes that may be regulated by RofA, and the identification of other factors that interact with RofA in regulation, will be required to expand our understanding of RofA in the regulation of streptococcal virulence.

ACKNOWLEDGMENTS

We thank Patrick Piggot for his gift of pJM783. We are also grateful to Michael Gilmore, Doug Berg, and Robert Kranz for providing helpful advice. We thank David Haslam for his advice in the construction and purification of MBP fusion proteins.

This work was supported by Public Health Service grant AI38273 from the National Institutes of Health. M.G.C. is an Established Investigator of the American Heart Association.

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