Identification of *rocA*, a Positive Regulator of *covR* Expression in the Group A Streptococcus

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In the group A streptococcus (GAS; *Streptococcus pyogenes*), a two-component system known as CovRS (or CsrRS) regulates about 15% of the genes, including several important virulence factors like the hyaluronic acid capsule. Most of these genes, including *covR* itself, are negatively regulated by CovR. We have isolated two independent IS*S1* insertions in an open reading frame (ORF) that increases CovR expression as measured by a P*cov*-*gusA* reporter fusion in single copy in the GAS chromosome. This ORF, named *rocA* for "regulator of Cov," activates *covR* transcription about threefold. As expected, a *rocA* mutant is mucoid and produces more transcript from the *has* promoter since this promoter is repressed by CovR. This effect is dependent on the presence of a wild-type *covR* gene. In contrast to its activation of P*cov*, RocA negatively regulates its own expression. This autoregulation is not dependent on the presence of the *covR* gene. All the phenotypes of the *rocA* mutant were complemented by the presence of the *rocA* gene on a plasmid. The *rocA* gene is present in strains of all nine M serotypes of GAS tested and is absent from strains representing 11 other groups of streptococci and related bacteria, including strains of the closely related group C and G streptococci. It seems likely that *rocA* plays an important role in the pathogenesis of GAS since it affects expression of the global regulator CovR.

The human pathogen *Streptococcus pyogenes* (group A streptococcus [GAS]) is a gram-positive bacterium that causes a wide variety of diseases, including relatively mild and self-limiting infections of the throat and skin, such as pharyngitis and pyoderma, as well as life-threatening invasive diseases like septicemia, myositis, necrotizing fasciitis, and streptococcal toxic shock syndrome (for a recent review, see reference 8). The primary infection may also lead to serious sequelae like rheumatic fever, glomerulonephritis, and reactive arthritis (3, 5). In many cases, it appears that single GAS strains can cause all or most of these diseases.

Many virulence factors of GAS strains appear to be involved in the survival, spread, and persistence of the organism inside the human host. These are (i) factors that damage or degrade the host tissues, such as cysteine protease, hyaluronidase, pyrogenic exotoxins, streptokinase, and streptolysins; (ii) factors that are required for resistance to phagocytosis by the host immune system, such as M protein, C5a peptidase, and hyaluronic acid capsule; and (iii) proteins that promote adherence and colonization, such as M protein and protein F (for reviews, see references 1, 8, 48, and 55). GAS encounters distinct microenvironments during infection and probably responds to them by expressing genes that produce proteins necessary for infection at that particular site. The ability to differentially regulate a wide range of virulence factors is therefore likely to be essential for the success of GAS infections.

Two-component signal transduction systems, consisting of sensor kinases and DNA-binding response regulators, allow bacteria to respond differently to diverse environmental stimuli (27, 46, 57). In the sequenced M1, M3, and M18 GAS genomes, 13 two-component systems have been identified (19) and several of them have been studied in various GAS strains (17, 22, 26, 31). Among them, CovRS (also called CsrRS) is a major regulator, repressing at least seven known or presumed virulence factors, including the hyaluronic acid capsule synthesis operon (*hasABC*), pyrogenic exotoxin B (*speB*), the streptolysin S-associated operon (*sag*), streptokinase (*ska*), mitogenic factor/streptodornase (*speMF/sda*), and inhibitor of innate immunity (*mac*) (2, 17, 25, 33, 34). In addition, CovR represses its own expression (17). A recent analysis by microarray and real-time PCR indicates that CovR controls as many as 15% of the GAS genes, either directly or indirectly (22). Because this is such a large number of genes and because some genes controlled by CovR are themselves known or suspected regulators, it seems likely that CovR is involved in a regulatory cascade.

Response regulators usually act by binding to DNA in the vicinity of promoter sequences. This is true for CovR as well, since recent in vitro studies have shown that CovR binds near several *covR*-regulated promoters (18, 41). For example, CovR binds to the promoter region of *hasA*, *sagA*, *ska*, *speB*, and *speMF* (18, 41). In vitro binding studies with the P*has* region identified five CovR binding sites, all of which are required for complete CovR-mediated repression in vivo (18). Thus, CovR appears to regulate the expression of some genes through direct interactions with their promoter regions.

Although we have begun to understand how CovR regulates the expression of different virulence genes, regulation of *covRS* expression is not well understood. It is known that CovR negatively regulates its own transcription (17) and that Rgg, a transcriptional regulator, activates *covRS* transcription (7). In

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addition, *covR* expression is also regulated by growth conditions. For example, *covRS* transcription reaches its maximum during the exponential growth phase and declines as the stationary phase begins (17, 41). Nutritional conditions, such as amino acid starvation, also affect *covRS* transcription by some unknown mechanism (54). Therefore, it appears that expression of the *covRS* operon may be controlled by a complex regulatory network.

The goal of this investigation was to isolate a regulatory factor(s) that modulates the expression of the *covRS* operon. In the present work, we report the identification and characterization of a gene that activates *covRS* expression. Because it activates *covR*, we named this gene *rocA* (for "regulator of CovR"). Inactivation of *rocA* in GAS results in decreased *covR* expression and derepression of *has* transcription.

MATERIALS AND METHODS

Bacterial strains and media. GAS (*S. pyogenes*) strain JRS4 is a streptomycin-resistant derivative of serotype M6 strain D471 (50). The GAS strain RTG229 was derived from JRS4 and contains a copy of the Tn*916*-J4 transposon on the chromosome, which provides homology for recombination of the pVIT plasmids as described previously (6, 20). All GAS strains described here are derivatives of JRS4 and RTG229. *Escherichia coli* XL1-Blue (Stratagene) and TG1 Rep (47) were used as the hosts for plasmid construction and were grown in Luria-Bertani broth with agitation (49). GAS strains were grown at 37°C without agitation in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY). Antibiotics were used at the following concentrations: ampicillin at 100 μg/ml for *E. coli*, chloramphenicol at 30 μg/ml for *E. coli* and 10 μg/ml for GAS, erythromycin at 500 μg/ml for *E. coli* and 1 μg/ml for GAS, kanamycin at 50 μg/ml and 200 μg/ml for GAS, and spectinomycin at 100 μg/ml for both *E. coli* and GAS.

Construction of the pVIT GAS strain used to measure Pcov expression. The VIT system was used to deliver the Pcov-*gusA* reporter fusion in single copy to an ectopic chromosomal location to study Pcov expression (6). The pVIT plasmid contains regions of homology to the resident Tn*916*-J4 transposon on the RTG229 chromosome. A DNA fragment containing the Pcov-*gusA* reporter was cloned into the homologous region of pVIT for integration into the chromosome.

The *covR* promoter region used for translational fusions was amplified from the JRS4 chromosome by using primers R1-CovOrf1 (gaattcTGGTATTAGTTTTAGACAAAGACGC) and Cov-GusAS (tctaga**TGTCATT**TATACCAACCCTTATCCTCTTA). The restriction site at the 5′ end of each primer is indicated by lowercase letters, the sequence homologous with the chromosome is indicated by uppercase letters, and the first two codons of the *covR* open reading frame (ORF) are in boldface. The 504-bp amplified fragment, which contains the *covR* promoter, untranslated sequence, and two codons, was blunt end ligated into *Sma*I-digested pBluescript II KS (Stratagene) to generate pEU7415. To clone the *gusA* reporter gene in frame with *covR*, we used the *Xba*I site that was inserted into the primer.

The *gusA* gene (1.8 kb) was amplified from plasmid pNZ8008 (9) by using primers GusL (gctctaga**ATG**TTACGTCCTGTAGAA) and GusR (gctctagaT CATTGTTTGCCTCCCTG) and was restricted with *Xba*I (site synthesized into the primer; underlined). The *Xba*I fragment containing the *gusA* gene was ligated to *Xba*I-restricted pEU7415 to create pEU7416. The orientation of *gusA* and the in-frame fusion of the Pcov-*gusA* gene were verified by sequencing.

To insert the Pcov-*gusA* reporter fusion at the pVIT locus, plasmid pVIT164 (20) was used. A *Pst*I-*Sac*I fragment from pEU7416 carrying Pcov-*gusA* was cloned into *Pst*I-*Sac*I-digested pVIT164 to generate pJRS2227. Plasmid pJRS2227 was linearized with *Pvu*II and transformed into RTG229 to produce JRS2227 as described previously (6). JRS2227 is kanamycin resistant and erythromycin sensitive, and the presence of both junctions of the Pcov-*gusA* fusion at the pVIT locus was verified by PCR.

Isolation of *rocA* mutants. The procedure described by Maguin et al. (39) was used to generate insertion mutants of GAS. Briefly, JRS2227 was transformed with pGhost9::ISS1 and transformants were selected on THY agar containing kanamycin and erythromycin at 30°C. An overnight culture was made from a single transformed colony at 30°C with erythromycin. Cultures were diluted 100-fold in the same medium, grown for 2 h at 30°C, and then shifted to 37°C for 2.5 h to select for transposition. Insertion mutants were selected on THY agar containing kanamycin and erythromycin at 37°C.

The location of the inserted ISS1 element was identified by one of two methods. A template generated by self-ligation of *Hin*dIII-digested chromosomal DNA was subjected to inverse PCR by using primers GATTGTAACGTAGAT AATAACCAACAGC (ISS1Rout1) and GCAAGAACCGAAGAAATGGA ACG (ISS1Fout1). The PCR product was sequenced with primer AATAGT TCATTGATATATCCTCGCTGTCA (ISS1-Rout2) to identify the flanking sequences. Alternatively, the self-ligated product was used to transform *E. coli* TG1 and erythromycin-resistant transformants were selected. Plasmid DNA containing flanking sequence was isolated and sequenced with primer GGTCTTAATG GGAATATTAGC (ISS1-For4) as previously described (13). The flanking sequences were identified by comparison to the M1 and M5 genome sequences.

Colonies in which the plasmid vector sequence had been excised from the chromosome were obtained by growth at 30°C without antibiotic selection, which permits plasmid replication (39). Erythromycin-sensitive (Em^s) colonies, indicating that the plasmid had been excised, were selected by plating on solid medium at 37°C. Em^s colonies were confirmed for loss of the plasmid sequence by PCR with primers homologous to the flanking regions.

Insertional inactivation of *rocA*. To create plasmids for inactivation of the *rocA* gene, a fragment internal to the coding region was amplified from JRS4 with high-fidelity Herculase polymerase (Stratagene) with primers AGCATTCAAG ATACCTTCATAAGTAAGTCT and TCCACTAGTGTCTATTAGTTTGGT TAAGCG. The amplified fragments were blunt end ligated to *Sma*I-restricted pUC-Spec (28), a suicide vector unable to replicate in GAS, to create pEU7457 (Fig. 2). The amplified internal *rocA* fragment was also blunt end ligated to *Sma*I-restricted pSK-Erm (a pBluescript SK derivative in which the ampicillin resistance gene has been replaced with the erythromycin resistance gene from Tn*1545*; I. Stojiljkovic, unpublished data) to generate pEU7460 (Fig. 2). Plasmid pEU7457 was introduced into JRS2227 by electroporation, and transformants were selected on THY agar with spectinomycin. A spectinomycin-resistant transformant resulting from homologous single-crossover integration of pEU7457 into the GAS chromosome and inactivation of the *rocA* gene was named JRS2268. The plasmid-chromosome junctions of JRS2268 were verified with the following primer pairs: 5′ junction, ccgaattcCTGATTTAATCTTTTAAGCTGT (RocAS1; see Fig. 2, arrowhead 1) and ggaaacagctatgaccatg (M13Rev; see Fig. 2, arrowhead 2); 3′ junction, ccgaattcATTAGTAATAGATTAACATATT (RocAA1; see Fig. 2, arrowhead 4) and tgtaaaacgacggccagtg (M13For; see Fig. 2, arrowhead 3). The *rocA* gene was also inactivated in strains JRS4, JRS948 (17), JRS 964, JRS965, and JRS966 (11, 18) by transformation with pEU7460 and selection for erythromycin-resistant transformants. This generated JRS2278, JRS2279, JRS2351, JRS2285, and JRS2286, respectively. These strains, in which *rocA* was inactivated, were verified by PCR across the plasmid-chromosome junctions by using primer pairs RocAS1 and M13For for the 5′ junction and RocAA1 and M13Rev for the 3′ junction.

Construction of the *rocA*-complementing plasmid pJRS2266. A PCR fragment containing all of *rocA* and 330 bp upstream of its start codon was amplified from JRS4 by using primers RocAS1 and RocAA1, which introduced unique *Eco*RI sites at both ends. The resulting 1.78-kb fragment was digested with *Eco*RI and ligated into *Eco*RI-digested pLZ12 (11), a chloramphenicol resistance-encoding natural shuttle vector able to replicate in GAS, to create pJRS2266.

Deletion of the *covR* gene. Plasmid pEU7529 was used to delete *covR* from the GAS strains (A. Gusa and J. R. Scott, unpublished). This plasmid was constructed from pJRS943 (17), which contains, in addition to the *covR* ORF, the upstream and downstream regions. Plasmid pJRS943 was restricted with *Bsg*I and *Xcm*I to delete *covR* and blunt ended (17). A spectinomycin resistance-encoding cassette was isolated by *Sma*I restriction from pSL60-I (37) and ligated to *Bsg*I-*Xcm*I-restricted and blunted pJRS943 to create pEU7521. The ampicillin resistance gene from pEU7521 was removed by *Pvu*I digestion, followed by self-ligation, to generate pEU7529. Plasmid pEU7529 was linearized by *Sac*I and transformed into JRS2227 and JRS2281. Spectinomycin-resistant transformants containing the *covR* deletion were selected and were confirmed for the replacement of the *covR* gene by PCR as previously described (17).

RNA blot analysis. GAS strains were cultured in THY, and growth was monitored by using a Klett-Summerson colorimeter with a red filter. Total RNA was isolated from different phases of growth as described previously (4), except that glycine was omitted from the medium. RNA was pelleted by sedimentation through 5.7 M CsCl (40) or by FastPrep (Bio 101) (4) as described previously. DNase I-treated RNA was assayed on Zeta-Probe membranes (Bio-Rad) as previously described (4). DNA probes were prepared by PCR amplification with the JRS4 chromosomal DNA as the template. The primer pairs used in this study are the following: *covR*, CAGCTATTCTTTGATATACTCTTTAGAG (5Cov) and Cov-GusAS; *gyrA*, GATCTGCAGGAATACGACTCATTTCTCTTTAT CCC and GTCATCCTGACCGCTTGTCAAAAGG; *hasA*, GAGAGAGAATT CACCTAGGAGTGTTTGATTTT (intHasA1) and GAGGAGGAATTCAGA
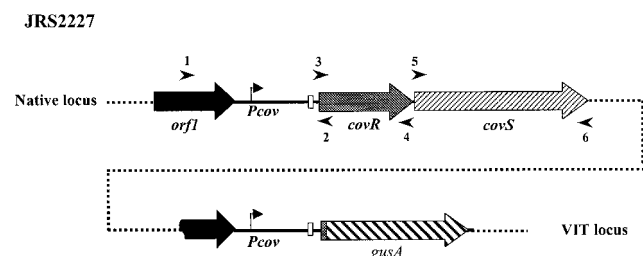
JRS2227

FIG. 1. Construction of P*cov*-*gusA* reporter strain. The promoter region of *covR*, along with the sequence encoding the first two amino acids of CovR (amplified by primers 1 and 2 [arrowheads]), was fused to the *gusA* reporter gene isolated from plasmid pNZ8008. The P*cov*-*gusA* reporter construct was inserted into the GAS chromosome at the VIT locus, which is not linked to the native *covRS* locus. Native *covRS* and the region of *covR* that is present at the VIT locus are shown. Symbols: bent arrow, *covR* promoter; white box, ribosome binding site of CovR; checkered box in front of *gusA*, first two amino acids of CovR; broken black arrow, part of the upstream ORF present in the P*cov*-*gusA* fusion. Arrowheads indicate primers used for PCR analysis. The orientation of P*cov*-*gusA* relative to the native *covRS* locus is unknown.

TGCCGAGTCATTA (intHasS1); *sagA*, GGAGGTAAACCTTATGTTAA (SagAL) and AGATTATTTACCTGGCGTAT (SagAR).

GusA assays. For plate assays, 10-μl volumes of overnight GAS cultures were spotted onto THY agar plates containing 200 μg of X-Glu (Gold Biotechnology Inc.) per ml. GAS strains that produced low GusA activity were white, while strains that produced high GusA activity were blue. Specific activity of GusA was assayed from GAS cultures grown to late exponential phase as previously described (14). For some experiments, GAS cultures were grown in THY broth buffered with 100 mM Tris-HCl at pH 7.2. The rate of hydrolysis was standardized by comparison to known concentrations of glucuronidase (Sigma catalog no. 500-0006). One unit of GusA activity was defined as that which liberates 1 μg of phenolphthalein (from phenolphthalein glucuronide)/h/mg of protein of GAS lysate at 37°C, pH 6.8. The protein concentration was determined by the Bio-Rad protein assay standardized against bovine serum albumin.

RESULTS

Construction of a P*cov*-*gusA* reporter strain and isolation of *rocA* mutants. To facilitate studies of the regulation of the CovRS operon, we constructed a P*cov*-*gusA* reporter strain. The entire promoter region of *covR*, including the untranslated leader sequence and the first two codons of the *covR* ORF, was fused to a *gusA* reporter gene to create a translational fusion. By using the pVIT system, this P*cov*-*gusA* reporter fusion was then integrated at an ectopic location on the chromosome of strain RTG229 (20), an M6 GAS strain derived from JRS4, to generate JRS2227 (Fig. 1). In this strain, the native *covRS* region remains unaltered.

We used insertional mutagenesis to identify potential activators of *covR* expression. For this purpose, we used the insertion sequence IS*S1* because it appears to insert itself randomly into the genome of gram-positive bacteria, including various streptococci (39, 59), and because it rarely inserts itself more than once into the same cell (39, 51, 59). We introduced this transposon into JRS2227 on pGhost9::IS*S1*, a plasmid whose replication is temperature sensitive (38). An erythromycin-resistant (Erm^r) transformant containing pGhost9::IS*S1* was grown overnight at 30°C, and Erm^r colonies containing the transposon were isolated at 37°C. In three different experiments, the transposition frequency (as measured by the number of Erm^r colonies divided by the total number of colonies at 37°C) was between 0.3 and 0.5%, which is comparable to frequencies obtained for IS*S1* transposition in other streptococci (51, 59, 60).

Among the operons that are negatively regulated by CovR is the *has* operon, required for production of the hyaluronic acid capsule (17). We reasoned that inactivation of an activator of P*cov* would cause increased transcription of P*has* because there would be less CovR expression. This would result in a mucoid colony phenotype in the JRS4-derived GAS strain JRS2227, which produces very little capsule. Therefore, we screened the JRS2227 colonies containing inserted IS*S1* for a mucoid phenotype. Mucoid colonies were then tested by a plate assay by using X-Glu to monitor expression of GusA from the P*cov*-*gusA* reporter fusion in the strain. A defect in expression of P*cov* results in production of white colonies. Among the mucoid colonies, approximately 30% were white.

To eliminate any possible insertion in the *covRS* operon, the size of the *covRS* region was verified by PCR amplification with primers 1 plus 2, 3 plus 4, and 5 plus 6 (Fig. 1), which include the region upstream of the *covRS* operon, as well as the entire *covRS* operon. The clones that showed wild-type-size fragments, suggesting an intact *covRS* region, were chosen for further analysis.

The site of the IS*S1* insertion was identified as described in Materials and Methods for 31 of the mucoid white colonies. In all 31 cases, Southern hybridization analysis showed that IS*S1* insertion had occurred at only one location. Among these mutants, more than one independent insertion had occurred in eight different genes. Four of these genes (*SPy1032* [*hylA*], *SPy481*, *SPy1505*, and a conserved ORF [*SPyM18_0587*] absent from the M1 and M3 genomes but present in the M5 and M18 genomes) had multiple insertions in the same location and thus could be sisters. Multiple independent insertions at different locations were found in each of the four remaining genes, *SPy 1981* (*relA*), *SPy1605*, *SPy159*, and *SPy0534* (*aroE*). Although none of the genes into which IS*S1* had inserted itself showed homology to any known transcriptional regulator, *SPy1605* showed some homology to sensor kinases (see below), which are involved in gene regulation. Therefore, we focused our studies on *SPy1605*, which we call *rocA* (for "regulation of *covR*"; see below). The *rocA* gene encodes a polypeptide of 451 residues. Two independent IS*S1* insertions had occurred in this gene and were located within codons 25 and 91, respectively. The *rocA* gene is preceded by a putative promoter region upstream of the translational start codon and followed by a potential rho-independent terminator just downstream of the stop codon (Fig. 2A). The gene downstream of *rocA* is transcribed in the opposite direction, which makes it unlikely that the IS*S1* insertions would have polar effects.

RocA regulates the expression of *covR*. To be sure that the phenotype of the *rocA* insertion mutation did not result from additional spontaneous mutations elsewhere in the genome, we inactivated the *rocA* gene in the nonmutagenized P*cov*-*gusA* reporter strain JRS2227. A PCR-amplified fragment internal to the coding region of *rocA* was cloned into the suicide vector pUC-Spec (28) to produce pEU7457. Following introduction of this plasmid into JRS2227, recombinants in which pEU7457 had been integrated into *rocA* were selected as Spec^r colonies. This integration results in inactivation of the *rocA* gene (Fig.
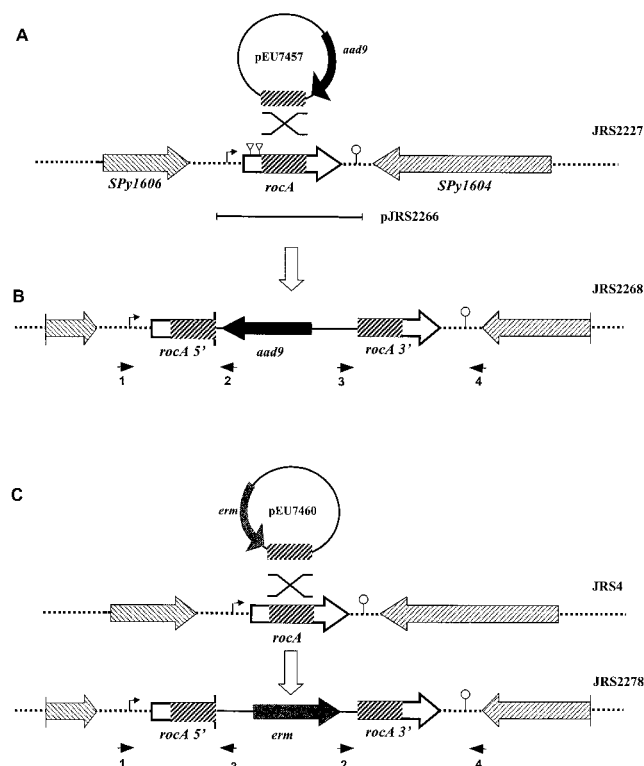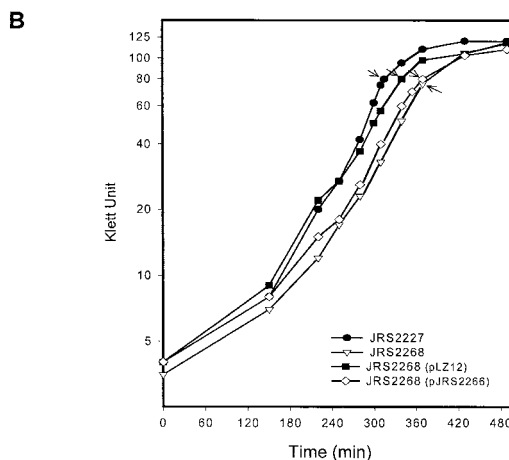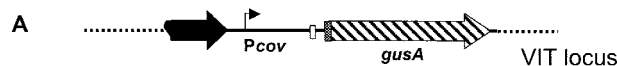
FIG. 2. Construction of a *rocA* null mutant. (A) Region surrounding *rocA* in the chromosome of GAS. Plasmid pEU7457 (circle), which was used for insertional inactivation, contains a region internal to the *rocA* ORF (thick hatched box) and contains *aad9*, which encodes spectinomycin resistance (thick black arrow). The direction of transcription of the ORFs flanking *rocA* is indicated by striped arrowheads. The region of *rocA* that was cloned in complementing plasmid pJRS2266 is indicated by a bar below the chromosome. (B) JRS2268 was produced by homologous recombination (indicated by the X above the representation of the chromosome in panel A), which inserted pEU7457 into the wild-type *rocA* gene in the JRS2227 chromosome. (C) JRS2278 was produced by a similar targeted insertion in JRS4 by using integrational plasmid pEU7460, which contains *erm*, encoding erythromycin resistance (gray box), and a region internal to the *rocA* ORF (thick hatched box) as shown. Symbols: dotted lines, chromosome; striped boxes, coding regions with the directions of transcription indicated by the arrowheads; bent arrow, putative promoter; triangles, IS*S1* insertion sites; lollipop, putative rho-independent transcription terminator. Small arrowheads below the chromosome represent primers used to confirm plasmid insertion into the chromosome (see Materials and Methods). The figure is not drawn to scale.

2B). In these integrants, the junctions between the integrated plasmid and the chromosome were identified by PCR analysis (by using primers 1 plus 2 and 3 plus 4 [Fig. 2B]). In addition PCR with primers 1 plus 4 failed to amplify a full-length copy of *rocA*.

Transcription of P*cov* was quantitated in these strains by measuring the activity of GusA produced from the P*cov*-*gusA* reporter fusion (Fig. 3A). For this assay, cells were grown in THY broth and harvested late in the exponential phase (Fig. 3B). We found that the *rocA* mutant strain (JRS2268) showed threefold less P*cov*-*gusA* expression than its *rocA*+ parent (JRS2227, Fig. 3C). To be sure that this effect was caused by the *rocA* mutation, we used complementation. We cloned a

FIG. 3. Expression of the *covR* gene in the wild-type and *rocA* mutant strains. (A) A *gusA* reporter system was used to measure the expression from the *covR* promoter at the VIT locus (symbols are as in Fig. 1). (B) Arrows indicate the times at which the samples were assayed for GusA activity. (C) The values shown are units of glucuronidase activity (with standard errors of the mean of experiments repeated at least four times). The relative ratio of GusA activity with respect to that of the wild type (WT) is shown.

	GUS (U/mg) (P*cov*-*gusA*)	Gus Relative to WT
JRS2227 (*rocA*+)	65 ± 5	1.00
JRS2268 (*rocA*)	21 ± 4	0.33
JRS2268 /pLZ12 (*rocA* /*rocA*)	22 ± 4	0.35
JRS2268 /pJRS2266 (*rocA* /*rocA*+)	143 ± 22	2.20

DNA fragment including *rocA* with its potential promoter region into pLZ12, which replicates in GAS, to generate pJRS2266 (Fig. 2A). As a control, we used JRS2268 (*rocA*) containing the vector pLZ12. This control strain had the same GusA activity as strain JRS2268, while the complemented strain, JRS2268/pJRS2266, expressed about twofold more GusA than the parental *rocA*+ strain, JRS2227. Thus, when RocA was overexpressed from the multicopy plasmid, P*cov* expression was not only restored but was increased above the wild-type level (Fig. 3C). Therefore, it appears that RocA activates expression from the P*cov* promoter.

CovR is not required for the activation of P*cov* by RocA. Because CovR represses its own transcription by binding to DNA in the P*cov* region (Gusa and Scott, personal communication), it seemed possible that RocA could activate P*cov* by affecting the interaction of CovR with the P*cov* promoter DNA. If RocA acts on P*cov* through CovR, then in the *covR* *rocA* double mutant, P*cov* expression should be the same as in

TABLE 1. Regulation of *covR* expression by RocA is independent of functional CovR

Strain	Genotype	Gus activity (U/mg)[a]
JRS2227	*covR*+ *rocA*+	136 ± 23
JRS2281	*covR*+ *rocA*	50 ± 6
JRS2289	*covR rocA*+	332 ± 62
JRS2290	*covR rocA*	123 ± 10

[a] Regulation of *covR* expression was measured from the P*cov-gusA* fusion at mid-exponential phase. The values shown represent the average GusA activity of two independent experiments.

a *covR* single mutant. If RocA and CovR act independently on P*cov*, however, the decrease in transcription in the *rocA* mutant should be independent of the status of *covR* (mutant or wild type). To assay this, GusA specific activity was measured at the mid-exponential phase of growth. The results (Table 1) show a 2.4-fold decrease in P*cov* transcription in the *rocA* mutant compared to that in the *rocA*+ parent. The decrease was seen in both *covR*+ and *covR* mutant strains. Similarly, inactivation of *covR* resulted in a 2.7-fold increase in P*cov-gusA* transcription in both the *rocA*+ and *rocA* mutant strains. This strongly suggests that the two systems that regulate P*cov* are independent.

Effect of RocA on transcription of the *has* operon. Since the original *rocA* insertion mutants appeared mucoid, as expected of a strain deficient in an activator of P*cov*, we wanted to confirm that the increased mucoidy resulted from an increase in transcription of the capsule-encoding operon *hasABC*. To avoid any complications that might result from an ectopic reporter fusion assay, we measured *has* operon transcript directly. For this purpose, we inactivated the *rocA* locus in strain JRS4 by single-crossover integration of pEU7460 (Fig. 2C). The plasmid-chromosome junctions of the resulting *rocA* mutant strain, JRS2278, were verified by PCR (by using primers 1 plus 3 and 2 plus 4) (Fig. 2C) as described in Materials and Methods.

RNA was isolated from strains JRS4, JRS2278, and JRS2278/pJRS2266 in the mid- and late exponential growth phases (M and L, respectively, in Fig. 4A) and assayed by hybridization to a PCR-derived *hasA* probe. To ensure that equal amounts of mRNA from each strain were loaded on the filter, all samples were hybridized with a *gyrA* probe (Fig. 4B). We found that JRS2278 (*rocA*) had two- to threefold more *hasA* transcript than JRS4 at both stages of growth. This is as expected if RocA activates P*cov* to produce more CovR repressor. The complemented strain, JRS2278/pJRS2266, produced two- to threefold less *hasA* transcript than the JRS4 wild-type strain. This suggests, as above (Fig. 3), that *rocA* is overexpressed from pJRS2266, which results in overproduction of CovR for repression of P*has*.

Regulation of P*has* by RocA requires functional CovR. It seemed likely that RocA decreases P*has* transcription indirectly by activating P*cov* to produce more CovR. To study this, we used two separate approaches, one of which relied on GusA activity produced from a P*has-gusA* fusion and the other of which relied on direct measurement of the *hasA* mRNA level.

Federle and Scott (18) identified the binding sites at the P*has* promoter for CovR and constructed mutants with two
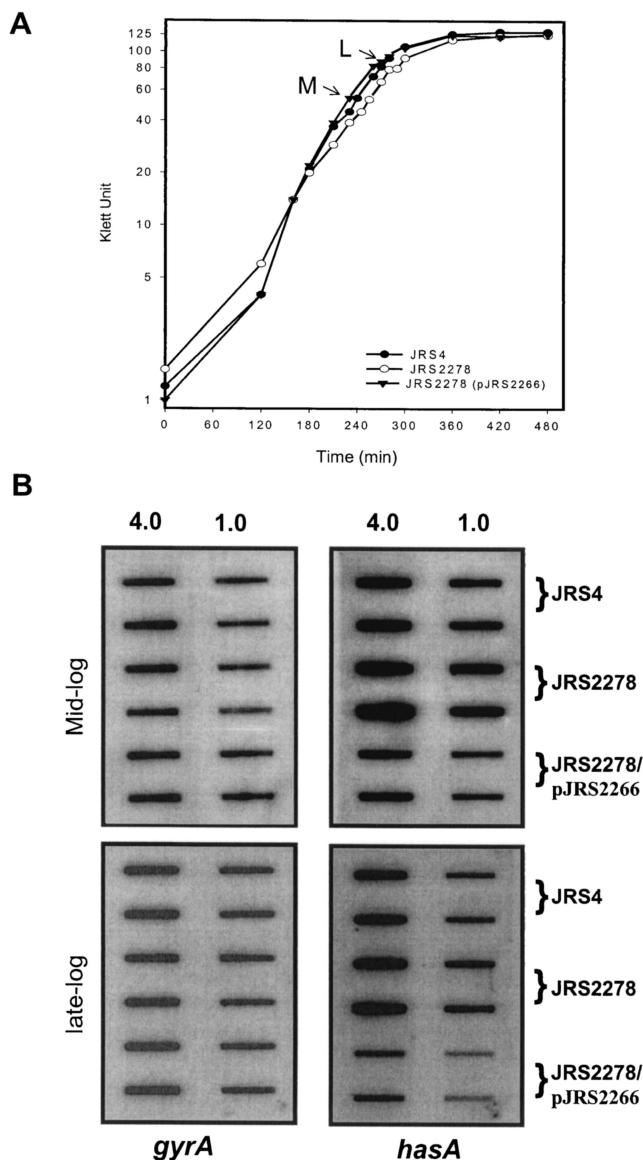

FIG. 4. Analysis of *hasA* gene transcription in the wild-type and *rocA* mutant strains. (A) Total RNA was isolated from JRS4 (wild type), JRS2278 (*rocA*), and JRS2278/pJRS2266 (*rocA/rocA*+) at the mid-exponential growth phase (M) and the late exponential growth phase (L). (B) On each filter, two dilutions (4.0 and 1.0 μg) of RNA were applied in vertically arranged duplicates. Membranes were probed with PCR-derived specific DNA probes internal to the coding region of *gyrA* and *hasA* (Materials and Methods). Results reported are representative of hybridization from three independent RNA isolations.

base substitutions at conserved T-T pairs at each of these sites. In two of these P*has* mutants, P*has* is still transcribed but is insensitive to repression by CovR. We reasoned that if RocA acts on P*has* indirectly through CovR, a *rocA* mutation should not affect transcription of the P*has* mutants that are not CovR regulated. In the P*has-gusA* fusion with no mutations in the promoter region, the *rocA* strain produces approximately fourfold more GusA activity than the wild type (Fig. 5A). In contrast, in the P*has-gusA* strain mutated at CovR binding site 1
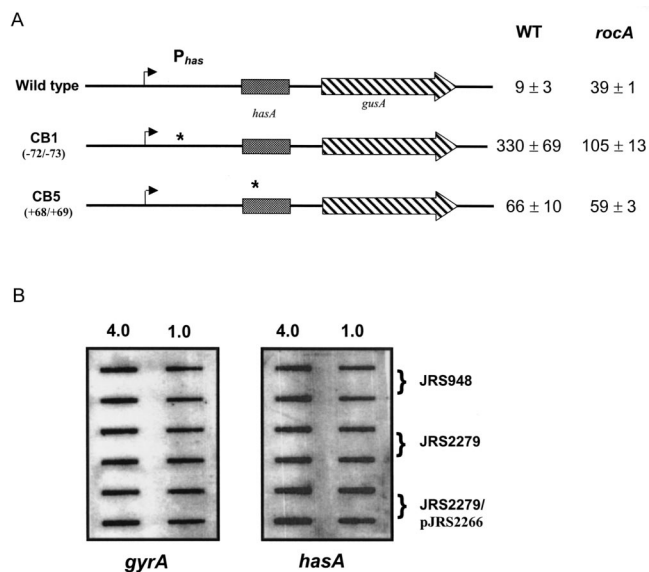
FIG. 5. Regulation of the *has* promoter by *rocA* requires functional CovR. (A). Stars indicate mutations in the *has* promoter region that make it insensitive to CovR repression. The name of the mutant is on the left, and the location of the mutation is in parentheses. The values shown are glucuronidase units with standard errors of the mean of at least four independent experiments. WT, wild type. (B) Transcription analysis of *hasA* by RNA hybridization. RNA was isolated at the mid-exponential phase from JRS948 (*covR*), JRS2279 (*covR rocA*), and JRS2279/pJRS2266 (*covR rocA*/*rocA*+). Blots were prepared and hybridized as described in the legend to Fig. 4. Results are representative of hybridization from two independent RNA isolations.
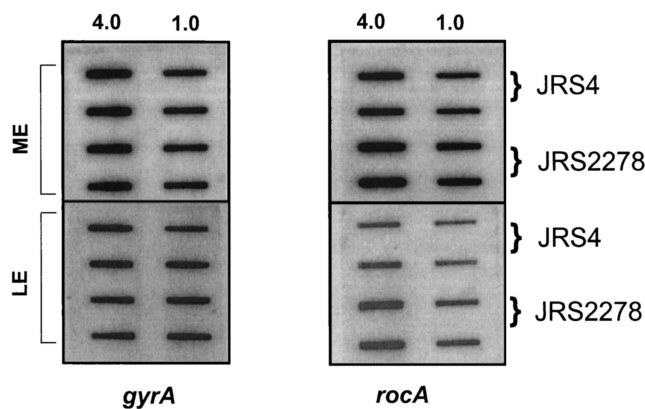

FIG. 6. RocA regulates its own expression. RNA was extracted from the JRS4 (wild-type) and JRS2278 (*rocA*) strains at the mid-exponential (ME) and late exponential (LE) phases as indicated in Fig. 4. Membranes were hybridized with *gyrA* and *rocA* probes. Blots were prepared and hybridized as described in Materials and Methods. The results shown are representative of hybridization from two independent RNA isolations.

(CB-1), there was no increase in GusA expression in the *rocA* mutant compared to that in the *rocA*+ strain. Instead, we observed lower GusA activity in the *rocA* mutant and the reason for this decrease is unknown. However, in the other P*has-gusA* strain, in which CB-5 is altered, mutation of the *rocA* gene did not change GusA specific activity (Fig. 5A). This implies that RocA probably acts on the P*has* promoter indirectly through CovR.

To confirm this observation, we measured the amount of *has* transcript. As shown above (Fig. 4), JRS2278 (*rocA*) shows about twofold more P*has* transcript than its *rocA*+ parent, JRS4. However, in JRS948 (*covR*), JRS2279 (*covR rocA*), and JRS2279/pJRS2266 (*covR rocA*/*rocA*+), the level of *hasA* transcript remained the same (Fig. 5B). This is consistent with the above result from the P*has* promoter mutants and suggests that the increased *has* expression seen in the *rocA* mutant is mediated by CovR. Therefore, it appears that RocA decreases P*has* transcription by activating transcription of P*cov* and producing more CovR.

RocA negatively regulates its own expression. Since it is not uncommon for transcriptional regulators to control their own synthesis, we used RNA hybridization analysis to test this for *rocA*. As before, we harvested RNA at the mid- and late exponential phases (Fig. 4A) and normalized to *gyrA* for a loading control on slot blots. At both stages of growth, there was two- to fourfold more *rocA* transcript in JRS2278 (*rocA*) than in JRS4 (*rocA*+) (Fig. 6). This suggests that RocA, which activates *covR*, negatively regulates its own expression (either directly or indirectly). Although *rocA* transcript appears more

plentiful in the mid-exponential phase than in the late exponential phase, autoregulation persists in the later stage of growth.

Autoregulation of RocA is independent of functional CovR. The RocA sequence contains no elements suggestive of a DNA binding protein, so its negative autoregulation is likely to be indirect. RocA activates transcription of CovR, and CovR is a negative regulator of many GAS promoters, so it seemed possible that negative autoregulation of *rocA* expression might also be mediated by CovR. To investigate this, we quantitated the *rocA* transcript levels in a *covR* mutant and its wild-type parent. The RNA was harvested in the late exponential and early stationary phases from cells grown in THY broth, and hybridization to an *rpsL* probe was used as a loading control. No difference was detected in the amount of *rocA* transcript in RNA from JRS4 (*covR*+) and JRS948 (*covR*) (Fig. 7). One of the genes repressed by CovR is *sagA*, which encodes streptolysin S (17), so an internal *sagA* fragment was also used to probe the same RNA to make sure the JRS948 strain had not mutated. As previously shown (17), JRS948 produces much more *sagA* transcript than its *covR*+ parent, JRS4 (Fig. 7). From these results, it appears that RocA negative autoregulation does not depend on the presence of functional CovR protein.

RocA is present in most GAS strains and not in strains of other streptococcal species. CovR is a major global regulator in all strains of GAS examined, and it appears from the above that RocA regulates the expression of CovR. Therefore, we investigated the prevalence of *rocA* in different GAS strains.

To investigate the distribution of *rocA* among the different streptococci, we used two approaches. First, a BLAST search with the *rocA* nucleotide sequence against the available GAS genome sequences showed that this gene is present in the sequenced M1, M3, M5, and M18 strains. A similar search found no significant homology to genes in any sequenced strains of other streptococcal species.

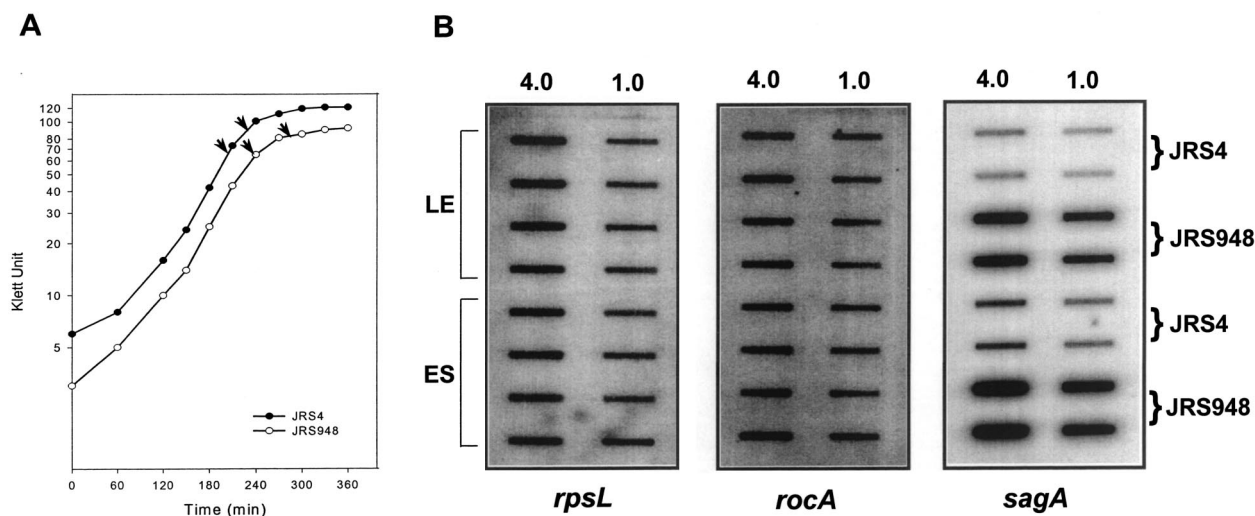To expand these observations, we used Southern hybridiza-

FIG. 7. Regulation of *rocA* expression by RocA is independent of functional CovR. RNA was extracted from the JRS4 (wild-type) and JRS948 (*covR*) strains at the late exponential (LE) and early stationary (ES) phases (arrows in panel A). Membranes were hybridized with *rpsL*, *rocA*, and *sagA* probes as described in Materials and Methods (B). The results shown are representative of hybridization from two independent RNA isolations.

tion to investigate the presence of a *rocA* homolog in strains representing nine of the multilocus enzyme electrophoresis types of GAS (42). For this analysis, a 1.78-kb fragment including the complete *rocA* gene was used as a probe and the hybridization was carried out under stringent conditions (Fig. 8). This probe hybridized with two *Hin*dIII fragments that differ in size and two *Hin*dIII fragments of constant size in all 13 GAS strains tested. The *Hin*dIII fragments of variable size indicate that regions flanking *rocA* vary among the GAS strains and correspond to the predicted sizes for M1, M3, M5, and M18 strains. The two constant *Hin*dIII fragments were ~0.92 and ~0.23 kb long. The 0.92-kb fragment is internal to *rocA*, and the 0.23-kb fragment includes 0.14 kb of upstream DNA. These results demonstrate that *rocA* is present in most or all strains of GAS.

On the other hand, the *rocA* probe failed to hybridize with DNA derived from representative strains of streptococcal group B, C, D, E, F, G, H, L, M, N, or O or with *S. pneumoniae*, *S. mutans*, *Enterococcus faecalis*, or *Lactococcus lactis* (data not shown). These results suggest that *rocA* is present only in GAS and is absent from all related bacteria, including the closely related streptococci of groups C and G.

DISCUSSION

In this study, we have identified a gene, *rocA*, that positively regulates *covR* expression. The P*cov-gusA* reporter gene fusion that we used to identify *rocA* is a translational fusion; therefore, the observed reduction of *gusA* expression in the mutant could be due to either transcriptional or translational regulation or both. However, when a P*cov-gusA* transcriptional fusion was used, 2.2-fold less Gus activity was obtained in the *rocA* mutant strain than in the wild type (data not shown). This indicates that *rocA* regulates P*cov* at the transcriptional level.

RocA appears to activate CovR expression, which acts as a repressor on several promoters. We found that the *rocA* mu-

tant strain is mucoid and shows more *has* transcription than the wild type (Fig. 4). This is expected, since in the *rocA* mutant, less CovR is produced, and as a result, *has* transcription is derepressed. In view of the fact that *has* expression was increased in the *rocA* mutant, we measured the transcription of genes, such as *sagA*, *ska*, and *speMF/sda*, that are normally repressed by CovR (17). We did not find any significant differences in transcription between the wild-type and *rocA* mutant strains (data not shown). It is possible that the effect of the *rocA* mutation on these genes was very small and that the RNA slot blot assay that we used to measure transcript levels is not sensitive enough to detect subtle changes. Alternatively, the *rocA* mutation may not affect the transcription of the *sagA*, *ska*, and *speMF* genes. It is possible that the amount of CovR present in the *rocA* mutant is sufficient for complete repression of the *sagA*, *ska*, and *speMF* promoters but that more CovR is required for complete repression of the *has* promoter. However, it is also possible that the active form of CovR that regulates the *sagA*, *ska*, and *speMF* genes is different from the one that represses the *has* promoter and that the latter form is affected by the *rocA* mutation whereas the former form is not.

The *rocA* gene encodes a polypeptide of 451 amino acid residues. A BLSLTP (version 2.2.3) search of the nonredundant GenBank database showed limited homology to various sensor kinase homologs, with probability values ranging up to e^{-37}. The homology was restricted to the C-terminal region of RocA, and the homology did not increase significantly when only the C-terminal 220 amino acids were used for a BLASTP search. Proteins that showed homologies to RocA of greater than e^{-10} include BlpH and ComD of *S. pneumoniae* (10, 24), AgrC of *S. aureus* (36), RgfC of *S. agalactiae* (52), and FasB and FasC of GAS (31), among others. Most of these sensor kinases are activated by a small peptide and are involved in quorum sensing (32).

Members of the sensor kinase superfamily exhibit clusters of highly conserved residues that are presumed to play crucial
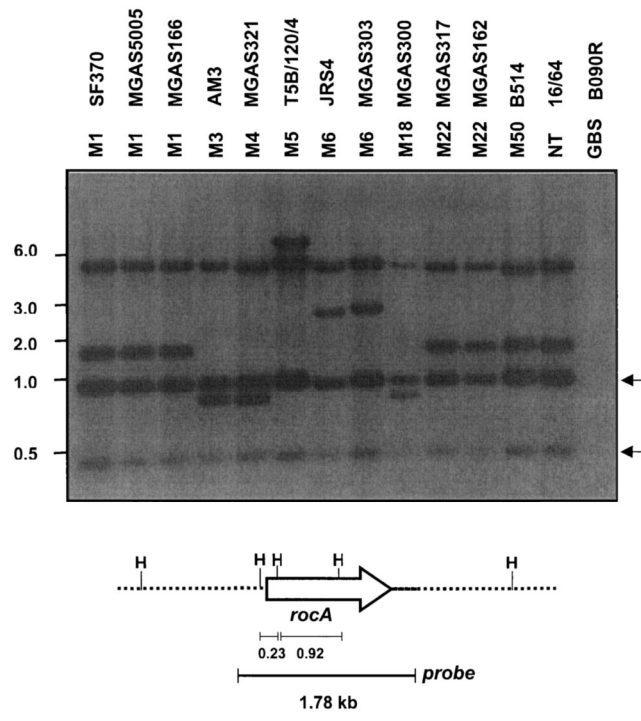
FIG. 8. Detection of the *rocA* gene by Southern hybridization. Genomic DNA from the strains was restricted with *Hin*dIII and separated in a 0.7% agarose gel by electrophoresis, and the presence of the *rocA* gene was detected by hybridization with a 1.78-kb fragment of the *rocA* region as shown at the bottom. M serotypes and strain numbers are indicated at the top. GBS, group B streptococcus; NT, nontypeable. Arrows indicate the constant fragments hybridized with the probe in all GAS strains. The values on the left are molecular sizes in kilobases.
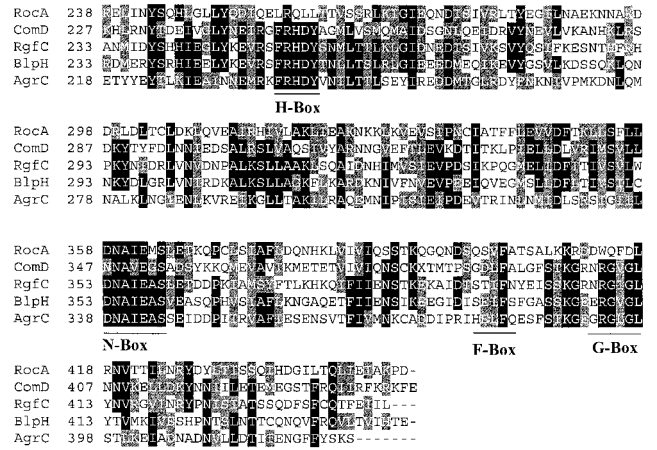

FIG. 9. Comparison of RocA with homologous proteins identified by a Blast-P search. The C-terminal regions of the different sensor kinases were aligned by using ClustalW. The GenBank accession numbers of the sequences are as follows: RocA, AAK34382; ComD, CAB39530; RgfC, AAM22582; BlpH, AAK74685; AgrC, AAB63269. Putative conserved boxes are indicated below the alignment.

roles in signal transduction. These characteristic sequence fingerprints have been termed the H, N, F, and G boxes (56, 57). The H box contains the conserved histidine residue that is involved in autophosphorylation. The other boxes are considered to be part of the kinase subdomain and are required for kinase activity (56, 57). In the RocA sequence, we did not find a conserved H residue in the region corresponding to the H box (Fig. 9). In fact, apart from the N box, no other conserved boxes were apparent (Fig. 9). This surprising deviation from the canonical homology boxes has been reported previously for some other sensor kinases (for a review, see reference 23). For example, sensor kinases in which the conserved histidine is replaced with arginine, aspartate, or tyrosine (23, 61) and sensor kinases in which various conserved boxes are absent (12, 30, 56) have been documented previously. Thus, if RocA encodes a sensor kinase, it belongs to an unorthodox family with variant phosphorylation and kinase domains.

The N-terminal regions of sensor kinases are highly diverse and contain hydrophobic domains required for membrane insertions, known as the transmembrane (TM) helices. There are usually two to eight TM helices that, along with the surface-exposed regions, play a crucial role in signal recognition. TMPRED (http://www.ch.embnet.org/software/TMPRED-form.html) and Goldman-Engelman-Steitz hydrophobicity (15) analyses of the deduced primary amino acid residues

predicted that the N-terminal 220 amino acids of RocA form seven TM helices with short (3- to 17-residue) interhelical regions. Thus, like sensor kinases, RocA is predicted to be membrane associated.

Analysis of the RocA sequence showed no obvious DNA binding motifs, such as zinc finger or helix-turn-helix motifs. In addition, a computer-based profile scan against the Pfam (http://us.expasy.org) protein families and conserved domain database (http://ncbi.nlm.nih.gov) did not show any domain related to DNA binding proteins. This strongly suggests that RocA is not a transcriptional regulator that binds directly to the promoter regions to modulate expression. Therefore, the regulation of P*cov* and P*rocA* promoters by RocA is likely to be indirect.

If RocA encodes a sensor kinase, it should have a cognate response regulator. In bacteria, the genes for cognate pairs of sensor kinases and response regulators are typically found together in a single operon. However, in the region containing *rocA*, there are no ORFs with homology to a response regulator. Although the presence of cognate response regulators at a different locus than sensor kinases is not common, it has been observed. For example, the genes for response regulators for *barA* of *E. coli* (43), *expS* of *Erwinia carotovora* (16), and *gacS* of *Pseudomonas syringae* (45) are all present far from those encoding the corresponding sensor kinases. Thus, if RocA is a sensor kinase, its cognate response regulator may be present elsewhere in the genome.

Since we observed both activation of P*cov* and repression of P*rocA* promoters by RocA (Fig. 3 and 6), RocA may act as a sensor kinase to stimulate either two different regulators with opposite functions or a single response regulator with different activities. There are several situations in which a single sensor kinase activates two different response regulators. For example, in *E. coli*, sensor kinase CheA interacts with CheB and CheY to regulate chemotaxis (35). On the other hand, response regulators with two opposite functions have also been documented. For example, in the *E. coli* osmosensing system,

response regulator OmpR functions both as an activator and as a repressor to differentially regulate the expression of the *ompC* and *ompF* genes (29, 44). Thus, whether RocA stimulates a single regulator with different activities or two different regulators remains to be explored.

RocA is present only in GAS, whereas the *covRS* system appears to be present in other streptococci as well, since *covRS* homologs have been found in group B streptococcus (21, 58), group C streptococcus (53), and *S. mutans* (GenBank accession no. AF393849). The streptococci that possess *covRS* but not *rocA* may modulate *covR* expression through a functional homolog with little or no primary sequence homology to *rocA*. Another possibility is that the activation of *covR* expression by RocA is unique to GAS. Perhaps, since strains of GAS are able to cause a very wide range of diseases, these bacteria require an additional level of *covR* regulation not required by other streptococci that have a more limited disease spectrum. The role of CovR in virulence gene regulation and the mechanism of regulation of *covR* expression have not been studied in other streptococci.

In conclusion, we have identified and characterized *rocA*, a novel regulatory gene involved in the activation of *covR* expression in GAS. Since CovR is a global regulator that controls 15% of the GAS genes, RocA may be involved in the regulation of a large network of genes. Moreover, RocA also regulates genes independently of CovR. Therefore, detailed characterization of RocA-mediated gene regulation may lead to a greater understanding of the events that occur during pathogenesis of GAS.

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