**Streptococcus pyogenes** CovRS Mediates Growth in Iron Starvation and in the Presence of the Human Cationic Antimicrobial Peptide LL-37

Barbara J. Froehlich, Christopher Bates, and June R. Scott*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

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We found that the global regulatory two-component signal transduction system CovRS mediates the ability of group A streptococcus (GAS) to grow under two stresses encountered during infection: iron starvation and the presence of LL-37. We also showed that CovRS regulates transcription of the multimetal transporter operon that is important for GAS growth in a low concentration of iron.

**GAS causes many types of disease.** *Streptococcus pyogenes*, the group A streptococcus (GAS), is a common and serious human pathogen that causes many different types of disease (12, 38). Among these diseases are streptococcal toxic shock syndrome and necrotizing fasciitis (2, 40, 52). Serious nonsuppurative sequelae can occur in some individuals following GAS infections, including acute rheumatic fever infections, chronic rheumatic heart disease infections (41), and acute glomerulonephritis (38).

To combat GAS infections, an understanding of the mode by which this bacterium initiates infection and of the ways in which it overcomes host defenses is important. GAS may enter through the oral mucosa to produce pharyngitis (“strep throat”), one of the most common childhood diseases. Although pharyngitis is usually a self-limited infection, annual direct costs associated with this disease are thought to be in the range of $1 billion in the United States (41). Another major route for GAS entry is the skin, usually following abrasions or insect bites. Local superficial infections may result in impetigo, deeper tissue involvement may manifest as cellulitis, and GAS may also become extremely invasive and lead to severe tissue destruction (necrotizing fasciitis) (38). Thus, this important human pathogen is capable of initiating infection in very different environments in its host, indicating its ability to grow in the face of very different stress conditions.

Although there appears to be some association of specific strains with particular diseases (5), some strains of GAS seem to be capable of causing most or all of the different types of disease discussed above. Therefore, we can conclude that interactions between the host and the pathogen are critically important in determining the outcome of infection. As an infection develops, GAS expresses different gene products in response to changes in the host microenvironment, which, in turn, is altered by the infection (43). For example, colonization of the skin (pH 5 [31, 57], usually at temperatures below 37°C, and a high concentration of salt if sweating occurs) probably requires different GAS factors than colonization of the oral mucosa (pH 6.7 and the presence of saliva) (1). If GAS invades different tissues, it may be faced with lower oxygen tension, different pH levels (pH 7.4 in blood), and a temperature of 37°C, or in the case of fever, even higher temperatures. The destruction of host tissue will alter the pH and other environmental conditions as well, and the production of an abscess results in a pH below 6.5 (7). Therefore, it seems likely that the regulation of expression of GAS virulence factors and even of “housekeeping” genes in response to changes in the host microenvironment plays a critical role in determining the specific type of disease that develops.

**Importance of CovRS in pathogenesis.** Although our understanding of GAS pathogenesis is not nearly as complete as that of the pathogenesis of many gram-negative bacteria, several transcriptional regulators have been identified in GAS, and their study is under way. Characterization has begun for several regulators specific for one or a few promoters (e.g., Nra and RofA, etc.) (6, 29, 33, 36), as well as more general ones, including the activator Mga (multiple gene regulator of GAS) (28). Among the 13 two-component signal-transducing systems (TCS) encoded in GAS genomes, the TCS CovRS (CsrRS) seems to play a central role in growth and pathogenesis (41, 43). In addition to regulating GAS gene expression in response to stress in laboratory settings (14), CovRS is required for biofilm formation (11). It also modulates the transcriptome during growth in human blood (22) and has been shown to be important in the development of GAS disease in animal models, including in cynomolgous macaques (56), and probably also in humans (53, 54). CovRS controls expression of about 15% of the GAS genome either directly or indirectly (21). Unlike the DNA binding protein (response regulator) of most TCS, the response regulator CovR represses most of the genes it controls (18, 20, 35, 48). It also represses its own transcription (23).

The GAS CovRS homologs in group B streptococcus, *Streptococcus mutans*, and *Enterococcus faecalis* have also been shown to regulate many virulence genes and to be important for pathogenesis in these organisms (6, 27, 32, 33, 55). Therefore, the development of a greater understanding of GAS CovRS might be helpful in elucidating the virulence of these additional human pathogens.

**CovRS mediates the stress response of GAS.** In most bacteria, response to environmental stress is mediated by secondary sigma factors, which are required for transcription of genes...
needed for growth in a stress environment. However, GAS does not encode homologs to stress response sigma factors. The only alternative sigma factor encoded in GAS genomes is homologous to the competence sigma factor of Streptococcus pneumoniae, and it is not expressed in laboratory growth conditions (44). Instead, we have found that CovS is required for GAS growth under general stress conditions (39°C, a pH of 6, or a high concentration of salt) (14). Under these stress conditions, genetic evidence indicates that CovS inactivates CovR and thus derepresses genes required for growth of the bacteria (13, 14). This work suggests that the environmental signal for the CovRS system may be envelope stress, as was found for the CpxRA TCS of Escherichia coli (15, 50).

Iron starvation stress. One of the important defenses of the host against infection is its ability to sequester iron. Like all bacteria, GAS requires iron for growth and has several mechanisms of competing with the host for iron acquisition. Because these mechanisms are energetically expensive and because a high iron concentration is toxic, the acquisition of iron by bacteria is regulated by several systems. These have only been partially characterized in GAS. Most intracellular iron in the host is stored as ferritin, or in heme complexes like hemoglobin and myoglobin, and by transferrin and lactoferrin. GAS can use heme as well as hemoglobin, myoglobin, heme-albumin, and myoglobin, and by transferrin and lactoferrin. GAS can take) (39). The multimetal transporter encoded by the mts operon as well as the sia operon (34), and a flu homolog called siu (streptococcal iron uptake) (39). The multimeric transporter encoded by the mts operon genes is also used by GAS to obtain iron (26).

To determine whether CovRS plays a role in iron starvation stress, as it does in other stresses it is likely to encounter during infection, we grew the GAS serotype M6 strain JRS4 and its ΔcovS and ΔcovSΔcovR derivatives at 37°C overnight in modified Z-THY (Todd-Hewitt broth supplemented with 2% yeast extract, 0.825 mM MgCl₂, 0.825 mM MnCl₂, 0.825 mM CaCl₂, and 0.825 mM ZnCl₂ and buffered with 100 mM HEPES to pH 7.4) (17). For iron starvation, 15 mM of the iron chelator nitrilotriacetic acid (NTA) was added to the medium at the time of inoculation. Growth of the overnight cultures was measured as the optical density at 600 nm (OD₆₀₀) and expressed relative to the OD of a control culture of the same strain grown in the absence of NTA. The experiment was repeated at least five times for each strain, and the results are presented in Fig. 1. We found that the ΔcovS mutant is significantly more sensitive to iron starvation than its wild-type parent, and this phenotype is complemented by the presence of the wild-type covS gene on a plasmid. In addition, we found that the plasmid with a covS H280A mutation, in which the phosphorylatable histidine has been replaced with alanine, is unable to complement the ΔcovS mutant in this experiment. The role of CovR in the GAS iron starvation stress response is indicated by the finding that the ΔcovR ΔcovS double mutant grows almost as well as its wild-type parent. Thus, as we previously found for GAS growth at a high temperature, low pH, and a high concentration of salt (14), it appears that CovS is needed to inactivate CovR for growth under iron starvation conditions. This suggests that one or more genes required for growth in low iron conditions are repressed by CovR.

Two transcriptional regulators of iron uptake genes have been identified in GAS: PerR and MtsR. In the presence of iron, MtsR represses transcription of the sia operon as well as its own transcription, (4) and in some strains, MtsR also represses transcription of the mtsABC operon (24), while PerR activates transcription of the mtsABC operon (47) (Fig. 2). CovR has not been identified previously as regulating iron uptake genes. Therefore, we used quantitative reverse transcription-PCR (RT-PCR) to compare the amount of the transcript in a ΔcovR strain and that of its parent for mtsR, mtsA, perR, flu/siu, and shr, the first gene in the sia operon. Cells grown in Todd-Hewitt broth supplemented with 0.2% yeast extract (THY) at 37°C were harvested in the log phase and in transition to the stationary phase. The RNA was pelleted by sedimentation through 5.7 M CsCl (37), and transcripts were quantified using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad) as described previously (49). The transcript levels were expressed relative to the amount of the proS transcript. Each experiment was repeated with at least two different RNA samples. We saw no difference between the wild-type
strain and the ΔcovR strain for shr, siu, perR, or mtsR. Because the amounts of the transcript for shr and siu were very low, as might be expected in the presence of iron, we are unable to determine whether they are regulated by CovR under these growth conditions. However, there was two- to threefold more mtsA transcript (relative to proS) in the ΔcovR mutant than in its wild-type parent strain in the transition phase of growth. In the log phase, this difference was slightly higher. The ΔcovS mutant showed no significant difference from its wild-type parent in the amounts of any of these transcripts, and the ΔcovR ΔcovS double mutant had two- to fourfold more mtsA transcript than its wild-type parent. Thus, it appears that CovR represses mtsA and that increased expression of mtsA may be required for growth of GAS in laboratory iron starvation conditions. It also suggests that iron starvation can be added to the list of stresses that cause CovS to inactivate CovR, presumably by favoring the phosphatase activity of CovS. This is the first indication that the global CovRS TCS has significant input to the iron uptake system of GAS and reemphasizes the pivotal role that CovRS plays in GAS growth and pathogenesis.

GAS growth in the presence of the innate-immunity cationic antimicrobial peptide LL-37. Another innate host defense mechanism is the production of the cationic antimicrobial peptide LL-37 from its precursor cathelicidin. The peptide is believed to permeabilize bacterial cell membranes, which leads to the death of the bacterial cell. In addition, there are many reports indicating that LL-37 serves as a chemottractant for leukocytes (9), as well as mononuclear cells and neutrophils (10), which eliminate the invading bacterial pathogens. This amphipathic 37-residue helical peptide is found throughout the human body, and its production in the skin has been shown to be triggered by the creation of a wound (16). Therefore, any successful pathogen with a route of entry through the skin must have a mechanism of defense against this peptide. For GAS, the role of the mouse analog of LL-37 in host resistance to a skin infection was demonstrated by Nizet et al. using knockout mice (42). They found that the lesions caused by GAS were larger in the wild-type mice than in the mutant mice lacking the gene for the antimicrobial peptide (CRAMP). In their work, a new GAS gene, ergR, which is in the Gmr family of transcriptional regulators, was shown to be important for the survival of the bacterium in the presence of LL-37, although neither the regulation of this gene nor its regulatory function in the GAS genome has yet been reported. Since the work of Nizet et al., indicates that the membrane-permeabilizing cationic antimicrobial peptide LL-37 provides an important element of the innate host defense system against GAS in skin infections, and since the CovRS system seems to respond to membrane stress, we wished to determine whether the bacterial response to this stress was mediated by the GAS global regulatory TCS CovRS.

Under several stress conditions, a ΔcovS mutant is unable to grow while a ΔcovS ΔcovR mutant grows well (14). To determine whether the same was true for the stress provided by LL-37, we compared the growth of the GAS serotype M6 strain JRS4 and its ΔcovS, ΔcovS ΔcovR, and ΔcovR derivatives. The GAS strains to be tested were grown overnight at 37°C in THY, diluted 1/20 in prewarmed (37°C) THY, and grown at 37°C until mid-log phase. The culture was then diluted 1/10 into prewarmed THY for growth in the presence of mature human cathelicidin LL-37 in 96-well plates (polystyrene with flat bottom; Costar 3598). To each well containing 1 volume of the diluted LL-37 in a final concentration of 0.2% bovine serum albumin, 4 volumes of the diluted bacterial culture was added, and the liquid was mixed well by pipetting it up and down. Serial twofold dilutions of LL-37 at concentrations from 250 μg/ml to 3.9 μg/ml were used. Each LL-37 concentration was tested at least in duplicate for every strain. Controls for each strain without LL-37 were always included to be sure the cells were growing in the plates. Growth at 37°C was monitored using a BioTek Synergy HT plate reader. The OD600 was measured every 20 min for at least 6 h. Just before each reading, the plates were shaken for 10 s at the lowest intensity on the plate reader.

The results, presented in Fig. 3, show that CovS is required for GAS growth in the presence of LL-37. Thus, LL-37 is similar to other stresses for GAS. To determine whether the requirement for CovS in the presence of LL-37 results from the CovS inactivation of CovR, we investigated the mutant that was deleted from both genes. In the absence of CovR, the CovS deletion mutant is less sensitive to LL-37 than the ΔcovS strain. This implies that, as is the case for other stresses, one or more genes required for growth in the presence of LL-37 are repressed by CovR.

However, the ΔcovS ΔcovR mutant is more sensitive to LL-37 than its wild-type parent, which differs from the findings above for iron starvation and all other stresses studied. Therefore, we investigated the effect of LL-37 on a ΔcovR mutant. We found that the single ΔcovR mutant grows as poorly as the ΔcovS ΔcovR double mutant in the presence of LL-37. The simplest interpretation of this is that CovR is needed for the activation (either directly or indirectly) of a gene(s) required for growth of GAS in the presence of the cationic antimicrobial peptide LL-37. We do not favor the alternative interpretation...
that CovS regulates a gene required for growth in the presence of LL-37 independently of CovR, i.e., by cross talking with another TCS response regulator, because CovS has not been demonstrated to have a CovR-independent function.

An operon activated by CovR (13; B. J. Froehlich and J. R. Scott, unpublished data), whose homolog in other gram-positive bacteria increases the resistance to cationic antimicrobial peptides (including the mouse analog of LL-37), is dnaT (8, 19, 30, 45, 46). The dnaT operon is responsible for \( \delta \)-alanilation of the anionic polymers lipoteichoic acid and wall lipoteichoic acid. A mutant deficient in dnaT, therefore, has a higher negative charge density in its cell wall, which may allow better binding of cationic antimicrobial peptides. Additionally, a dnaT mutation in \( B. subtilis \) alters expression of the surface stress-responsive two-component system liaRS and leads to the decreased production of the surface quality control proteases HtrA and HrtB (homologs of DegP of Escherichia coli) (25). Therefore, in GAS, the CovR-activated operon required for growth in the presence of LL-37 might be dnaT.

Growth of GAS under stress conditions (a low concentration of salt, pH 6, and 39°C) requires the inactivation of CovR by CovS. Expression of the CovR-repressed gene rsc is required for growth at 39°C, and rsc is derepressed by CovS when the strain is subjected to temperature stress (13). Since the gutR family gene crgR was found to be required for growth of GAS in the presence of LL-37 (42), it seemed possible that, like rsc, crgR might be repressed by CovR and derepressed in the presence of CovS under stress conditions (growth in LL-37). To test this possibility, we asked whether CovR represses crgR. RNA was collected from JRS4 and its \( \Delta \)covR mutant JRS948 at the exponential and transitional phases of growth, and quantitative RT-PCR was used to assay the crgR message. The results were normalized to the amount of proS mRNA in the same RNA preparation. From two separate RNA preparations in each growth phase, no difference in the amount of the crgR message was detectable (data not shown). We conclude, therefore, that the simple hypothesis is incorrect. Instead, it appears that one or more genes other than crgR, which are required for growth in the presence of the antimicrobial cationic peptide, are repressed by CovR. Because crgR is predicted to be a transcriptional regulator whose regulon has not yet been defined, further work is required to identify the Cov regulon genes(s) needed for GAS growth in the presence of LL-37.

**Conclusions.** Other bacteria that lack stress sigma factors, including the important human pathogen \( S. pneumoniae \) and the dental pathogens \( S. mutans \) and \( S. sanguinis \), encode CovRS homologs. In the case of \( S. mutans \), as well as in GAS (which has a CovRS homologous system as well as a stress sigma factor), this TCS has also been implicated in response to stress (51). Thus, an increased understanding of the CovS mediation of the stress response may also provide a model for the mechanism used in these other pathogens as well. The studies reported above were undertaken to determine whether CovRS is required for GAS growth under the additional stress conditions encountered during human infection. We found that in terms of stress provided by a low concentration of iron and by the presence of the human antimicrobial peptide LL-37, CovS is required for growth of GAS and that the deletion of CovR overcomes this effect. Thus, in addition to 39°C, pH 6, and a low concentration of salt, a low concentration of iron and the presence of LL-37 appear to stimulate CovS phosphatase activity so that CovS inactivates the CovR repression of genes required for growth.

The above work further supports the pivotal role CovRS plays in enabling GAS to grow under the many stress conditions that it encounters as it infects its host. A greater understanding of the CovRS system may suggest an effective approach to prevent this pathogen from causing serious human disease.

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**ADDITION IN PROOF**

While this paper was being reviewed, Wessels’ group published experiments extending our findings on LL-37 to additional strains of GAS (I. Grylllos, H. J. Tran-Winkler, M. F. Cheng, H. Chung, R. Bolcombe, W. Lu, R. I. Lehrer, and M. R. Wessels, Proc. Natl. Acad. Sci. 105:16755–16760, 2008). They found that addition of LL-37 to the medium increased the expression of several Cov-regulon virulence genes in a CovS-dependent manner. Although they did not address the role of CovR in this derepression, their results are completely consistent with our conclusion that growth with LL-37 produces membrane stress, leading to activation of the phosphatase activity of CovS, which results in derepression of Cov-regulon genes.

**REFERENCES**


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