Virulence control in group A *Streptococcus* by a two-component gene regulatory system: Global expression profiling and *in vivo* infection modeling

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Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved July 31, 2002 (received for review June 12, 2002)

Two-component gene regulatory systems composed of a membrane-bound sensor and cytoplasmic response regulator are important mechanisms used by bacteria to sense and respond to environmental stimuli. Group A *Streptococcus*, the causative agent of mild infections and life-threatening invasive diseases, produces many virulence factors that promote survival in humans. A two-component regulatory system, designated *covRS* (*cov, control of virulence; csrRS*), negatively controls expression of five proven or putative virulence factors (capsule, cysteine protease, streptokinase, streptolysin S, and streptodornase). Inactivation of *covRS* results in enhanced virulence in mouse models of invasive disease. Using DNA microarrays and quantitative RT-PCR, we found that CovR influences transcription of 15% ($n = 271$) of all chromosomal genes, including many that encode surface and secreted proteins mediating host–pathogen interactions. CovR also plays a central role in gene regulatory networks by influencing expression of genes encoding transcriptional regulators, including other two-component systems. Differential transcription of genes influenced by *covR* also was identified in mouse soft-tissue infection. This analysis provides a genome-scale overview of a virulence gene network in an important human pathogen and adds insight into the molecular mechanisms used by group A *Streptococcus* to interact with the host, promote survival, and cause disease.

microarrays | real-time RT-PCR | transcript analysis

Two-component gene regulatory systems composed of a membrane-bound sensor protein and a cytoplasmic response regulator are commonly used to regulate bacterial gene expression in response to environmental change (1). On stimulation by an extracellular signal, a histidine kinase domain of the sensor protein is autophosphorylated. Signal transduction occurs with transfer of the phosphoryl group to the cognate response regulator protein. Phosphorylation of the regulator protein alters its binding affinity for target promoter regions, thereby influencing the frequency of initiation of transcription of specific genes or operons and mediating a cellular response. Pathogenic bacteria often use two-component gene regulatory systems to control expression of genes encoding bacterial toxins, adhesins, and other virulence-associated molecules that interact with the host and promote survival *in vivo* (2).

Group A *Streptococcus* (GAS) is a Gram-positive human pathogen that causes mild throat and skin infections, such as pharyngitis and impetigo, and life-threatening invasive diseases including septicemia, myositis, cellulitis, streptococcal toxic shock syndrome, and necrotizing fasciitis (3). Rheumatic fever and poststreptococcal glomerulonephritis also can occur as serious sequelae of GAS infection. GAS encounter distinct microenvironments during host–pathogen interactions, and there has been considerable effort to identify regulatory mechanisms involved in coordinate expression of virulence determinants (4–11). When inactivated, the two-component regulatory system referred to as CovR-CovS (Cov, control of virulence; also known as CsrR-CsrS) resulted in a mucoid colony phenotype associated with overexpression of hyaluronic acid capsule (5). Transcripts encoding proteins likely to promote GAS survival and virulence in humans, including streptokinase, streptolysin S, and streptodornase (also known as mitogenic factor) also were more abundant in the mutant strain (7). *In vitro* phosphorylation-dependent oligomerization and differential binding of CovR to these target promoters has recently been demonstrated (12, 13). Consistent with increased virulence gene transcription and capsule production, the isogenic mutant strain is hypervirulent in mouse models of invasive disease and has enhanced resistance *in vitro* to complement-mediated opsonophagocytic killing by human polymorphonuclear leukocytes (5, 8, 14).

Despite the importance of two-component regulatory systems in microbial pathogenesis, little is known about the spectrum of genes that they control directly or indirectly. Moreover, even less is known about gene expression during GAS infection of a mammalian host. We addressed these issues by comparing the global gene expression of a WT serotype M1 GAS strain and its isogenic *covR* mutant derivative at three stages of growth *in vitro*. Transcription of select bacterial genes also was tested for *covR* regulation after mouse soft-tissue infection. These data provide a genome-wide view of a critical regulatory system influencing virulence gene expression in an important human pathogen.

Materials and Methods

Bacterial Strains and Media. WT MGAS5005 and its nonpolar isogenic derivative JRS950 (MGAS5005 *covR*) were cultured statically (37°C; 5% CO_2) in Todd Hewitt-0.2% yeast extract broth. Cells were harvested at mid-exponential (ME) (OD_{600} = 0.3), late-exponential (LE) (OD_{600} = 0.6), or stationary (S) (OD_{600} = 1.0) growth phases.

RNA Isolation from *in Vitro*-Grown GAS. Cell lysis and RNA isolation were conducted with a FastRNA kit (Q/BioGene, Carlsbad, CA) (15). RNA integrity was assessed with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Absence of

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: GAS, group A *Streptococcus*; Cov/*cov*, control of virulence; ME, mid-exponential; LE, late-exponential; S, stationary.

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contaminating DNA was confirmed by PCR and TaqMan real-time RT-PCR.

Microarray Hybridization. DNA microarrays were prepared as described (15). ORFs are designated with SPy numbers in accordance with the completed serotype M1 *Streptococcus pyogenes* SF370 genome (16). Hybridization experiments were performed with indirectly labeled cDNAs prepared from MGAS5005 and JRS950 by using an ARES DNA labeling kit (Molecular Probes). Expression ratios are representative of replicate experiments performed with RNA from independent GAS cultures.

Real-Time RT-PCR. Microarray data were confirmed for 102 genes with TaqMan assays as described (15). Triplicate assays were performed with RNA from at least two independent cultures. Transcription of gyrase subunit A (*gyrA*) was not affected by *covR* inactivation or under a variety of *in vitro* experimental conditions (M.R.G., L.M.S. and S. Reid, unpublished data; ref. 15); hence, *gyrA* (*spy1152*) expression was used to normalize *in vitro* TaqMan data. For analysis of *in vivo* transcripts derived from mouse extracts (see below), cDNA synthesis was primed with genome-specific primers (1,500 ng) and Superscript II according to the manufacturer (Invitrogen). Target detection (6FAM) was conducted in triplicate two-step multiplex RT-PCRs (20 μl) with TaqMan Universal PCR Master Mix on an ABI 7900HT (Applied Biosystems). Target C_t values were normalized to average C_t values for an internal reference transcript, 6-carboxy-4′,5′-dichloro-2′,7′-dimethylfluorescein-labeled prolyl-tRNA synthetase (*proS; spy1962*), which is not affected by *covR* inactivation and under different experimental conditions (M.R.G., L.M.S., and K.V., unpublished data).

Mouse Soft-Tissue Infection Model. Outbred, immunocompetent, hairless male Crl:SKH1-*hr*BR mice (5 wk; 20–25-g) (Charles River Breeding Laboratories) were used for s.c. inoculation of 0.1-ml pyrogen-free PBS or S phase (18 h) inocula of MGAS5005 or JRS950 (about 3×10^7 colony-forming units). At 2.5 days postinoculation the mice were killed and weighed, and the dermis and underlying soft tissues were harvested, foil-wrapped, and snap-frozen. Extracts (about 1 g) were pulverized and homogenized twice for 20 s in a FastPrep FP 120 (Q/BioGene) with 300 μl CRSR-Blue (Q/BioGene) and 300 μl acid phenol/chloroform, pH 4.3 (Sigma). Total RNA was purified by using RNeasy columns (Qiagen, Chatsworth, CA) and contaminating DNA was removed with DNase I (DNA-Free, Ambion, Austin, TX).

Methods are detailed in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Results

Inactivation of *covR* Alters the Global Gene Transcription Profile. Global gene transcription profiles were determined by DNA microarray analysis for a WT serotype M1 GAS strain and its isogenic *covR* derivative grown to ME, LE, and S phase (Fig. 1*A*). Consistent with data that CovR acts as a transcriptional repressor *in vitro* (5, 7, 8, 12), 98 and 229 transcripts were at least 2-fold more abundant in LE and S phase growth, respectively, in the isogenic *covR* mutant relative to the WT strain (Table 1 and Table 2, which is published as supporting information on the PNAS web site). Transcription of all five genes known to be controlled by *covR* [*hasA*, *sagA, sda* (*mf*), *ska*, and *speB*] was differentially repressed in the WT strain. Most genes showed the greatest differential regulation in S phase, a result that concurs with *covR* transcript accumulation in LE growth phase (7). Several broad categories of genes were regulated by CovR, including transcripts that code for membrane-associated transport proteins, proteins that participate in stress responses, regulatory proteins, and surface and extracellular proteins including putative virulence factors (Tables 1 and 2). Transcription

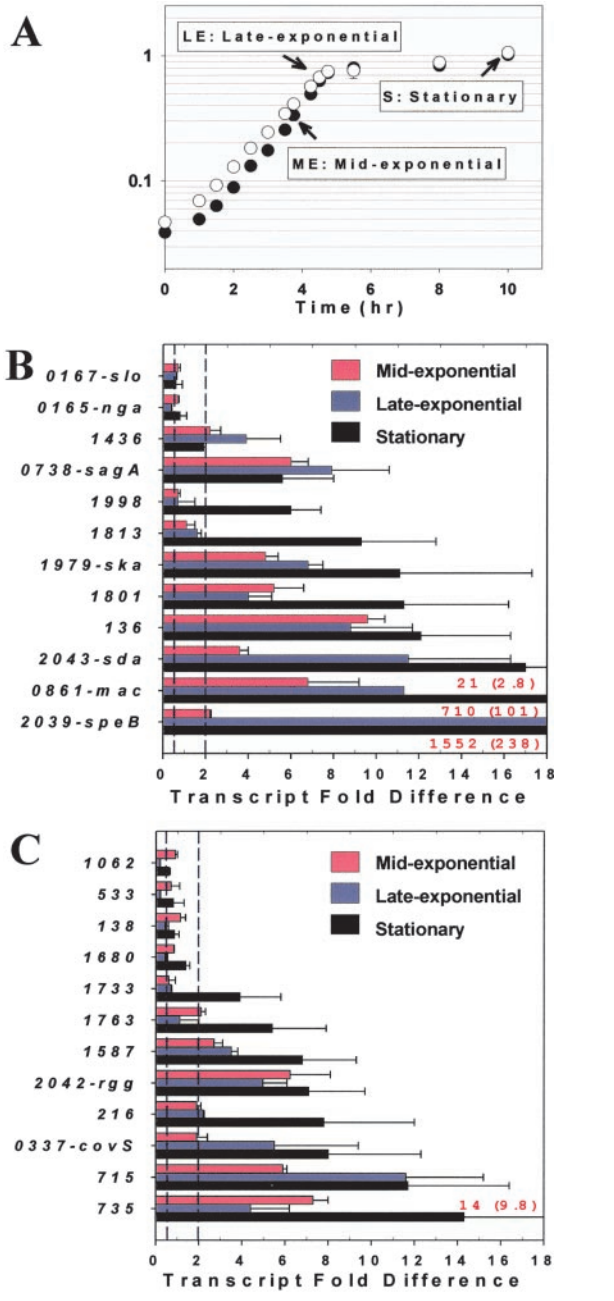

Fig. 1. Analysis of GAS *in vitro* transcript levels. (*A*) Growth curves for the serotype M1 GAS strain MGAS5005 (○) and its isogenic *covR* mutant derivative JRS950 (●) cultured statically in Todd Hewitt-0.2% yeast extract broth. Aliquots were removed for RNA extraction at equivalent OD_{600} values: ME, ≈0.3; LE, ≈0.6; and S, ≈1.0. (*B* and *C*) Relative abundance of CovR-regulated transcripts determined by TaqMan assays. Data shown are fold changes in transcript (*covR* mutant strain relative to WT) normalized to *gyrA* transcript amounts for selected genes. The values are averages obtained from analysis in triplicate of two independent RNA samples. Shown are data for genes encoding select extracellular proteins (*B*) and predicted transcriptional regulators (*C*). Transcript data (ME, pink; LE, blue; and S, black) are also presented in Table 2. The *y* axis labels denote SPy numbers assigned for serotype M1 GAS strain SF370 ORFs. Error bars represent SD. Dashed blue lines indicate 0.5- and 2-fold expression thresholds. Average fold differences (text) and SDs (parentheses) are shown for off-scale transcript data in red.

Table 1. Summary of gene categories with increased transcription in the GAS *covR* mutant strain, assessed by DNA microarray analysis

Functional classification*	ME	LE	S
Metabolism		(22)	(52)
Carbohydrate		6	17
Fatty acid/lipid			5
Nucleotide		9	13
Amino acid		5	11
Cofactor/vitamin		2	6
Information processing	(1)	(19)	(65)
Regulation		8	18
Protein biosynthesis	1	4	26
Replication/repair		4	14
Chaperones		3	7
Environment processing			
Transport/binding		16	34
Other†	(1)	(32)	(58)
Cellular division		1	1
Peptidoglycan synthesis		1	1
Extracellular proteins		9	15
Membrane proteins		11	18
Cytosolic proteins	1	10	23
Phage/mobile elements		9	20
Virulence factors‡		24	32
Total	2	98	229

Fold difference (mutant vs. WT) ≥ 2.0.
*Assigned by BLASTP and KEGG analysis.
†Characterized GAS and hypothetical proteins.
‡Includes known and putative virulence-associated genes assigned to another category in Table 1.

of 24 and 55 genes encoding proteins with inferred secretion signal sequences was CovR-regulated in LE and S phases, respectively.

Confirmation of DNA Microarray Data by TaqMan RT-PCR. To confirm the microarray data, relative transcript levels of selected genes were measured by reverse transcriptase TaqMan assays with RNA isolated from two independent ME-, LE-, and S-phase cultures (11). One hundred two genes demonstrating varying expression levels by microarray analysis were chosen for RT-PCR analysis because the proteins they encode may influence GAS-human interactions. The majority of genes identified as differentially expressed by microarray analysis were confirmed to have altered transcript levels by TaqMan analysis (Fig. 1 and Table 2). There were strong positive correlations ($r = 0.82$ for LE-phase results, data not shown; $r = 0.83$ for S-phase results, Fig. 2) between results obtained with the two techniques.

CovR Influences Transcription of a Large Array of Proven and Putative Virulence Genes. Inactivation of *covR* increases virulence, as assessed by mouse models of invasive disease (5, 8, 14). The enhanced-virulence phenotype has been attributed to increased production of antiphagocytic hyaluronic acid capsule and other proven and putative secreted virulence factors including streptolysin S (SagA), streptokinase (Ska), a nuclease (Sda/MF-1/SpeF), and an extracellular cysteine protease (SpeB) (5, 8, 11, 13, 14). In the *covR* isogenic mutant, 32 transcripts encoding known and putative virulence-associated proteins that may influence GAS virulence were more abundant (Table 1). For example, transcription of the *grab* gene (*spy1357*), which encodes a bacterial surface-anchored protein that binds with high affinity to host α_2-macroglobulin (17), was greatly increased (Figs. 1 and 2). Human α_2-macroglobulin inhibits proteolytic enzymes in the

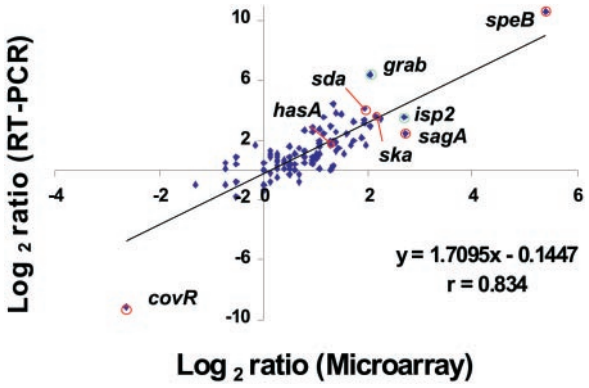

Fig. 2. Correlation of DNA microarray and TaqMan RT-PCR assays. The fold changes in transcript (*covR* mutant strain relative to WT) obtained by both methods for S-phase RNA were log-transformed, and the values were plotted against each other to evaluate their correlation. Select genes are shown for illustrative purposes.

kinin, complement, coagulation, and fibrinolytic pathways, thereby potentially benefiting GAS during infection.

Transcripts of the gene *spy0861* encoding a recently described novel secreted protein designated Mac or IdeS (18–20) also were more abundant in the *covR* mutant than the WT strain (Fig. 1). Mac is homologous to the α-subunit of human Mac-1, a leukocyte β2 integrin (αMβ2, CD11b/CD18, complement receptor 3) required for innate immunity among other host functions (21). Mac is expressed in human infections and inhibits reactive oxygen species production and opsonophagocytic killing of GAS (18–20).

Transcripts of genes encoding secreted proteins SPy1801 (Isp2) and SPy0136 (MspA) were substantially more abundant in the *covR* isogenic mutant (Fig. 1 and Table 2). The functions of these proteins are not known, but immunologic data show that they are produced during human infections (18).

Transcripts for two adjacent genes designated *spy2007* and *spy2009* that encode a laminin-binding adhesin (Lmb) homologue and an uncharacterized surface protein, respectively, also were more abundant in the *covR* mutant in the S phase (Table 2). In group B *Streptococcus*, *lmb* mutants show reduced adherence to immobilized laminin *in vitro* (22). Null or regulated expression of Lmb may decrease colonization of damaged epithelium but promote invasion of bacteria into the bloodstream (23). Terao *et al.* (24) recently reported that a homologous laminin-binding protein gene (*lbp*) is found in all GAS M types and that a *lbp* mutant adhered less efficiently to HEp-2 cells than the WT GAS strain, indicating that Lbp acts as an adhesin *in vitro*.

To summarize, we discovered by global expression analysis that CovR influences transcription of a large array of genes encoding proven and putative virulence factors. Our data suggest that the molecular mechanisms responsible for the enhanced-virulence phenotype of *covR* mutant strains are far more complex than previously thought.

CovR Affects Stress and Adaptation Responses. Stress and heat shock proteins comprise a significant proportion of bacterial gene products expressed *in vivo* for many bacteria, suggesting that they are involved in host–pathogen interactions (25). Many transcripts encoding predicted stress response proteins were more abundant in the *covR* mutant strain (Table 2). For example, transcripts of the *dnaK, dnaJ, grpE,* and *groEL* genes were increased in the *covR* mutant, primarily in S phase. These genes encode chaperonins that have a central role in stress responses in other bacteria. Convalescent sera from GAS-infected patients

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contain anti-DnaK and anti-GroEL antibodies, indicating that these proteins are made *in vivo* (18) and thus may be important in virulence. Transcripts encoding homologues of HSP-100/Clp ATPases (*spy0888*, *spy1509*, *spy2066*) also were increased in the *covR* mutant strain in S phase. Clp proteases (class III heat shock proteins) are highly conserved stress response proteins that repair or scavenge denatured proteins and modulate gene expression by influencing protein stability. Clp proteases also have been reported to enhance survival of pathogens such as *Listeria monocytogenes* (26) and participate in the virulence of *Staphylococcus aureus* (27, 28) and group B *Streptococcus* (22), suggesting a link between the bacterial stress response and survival in the infected host.

Small Number of Genes Down-Regulated in the *covR* Mutant Strain. Most bacterial two-component systems are known to activate gene transcription, but no genes are known to be activated by the CovR/CovS system. Consistent with CovR functioning mainly as a repressor, only 26 and four transcripts in LE and S growth phases, respectively, were reduced by at least 2-fold in microarrays in the *covR* mutant strain. Transcripts encoding proteins associated with carbohydrate and nucleic acid metabolism, nutrient transport or binding, protein synthesis, and peptide metabolism, were less abundant in the mutant strain (Table 2), suggesting that central metabolism was affected.

CovR Influences Transcriptional Regulators. The genome of GAS strain SF370 contains approximately 100 genes that encode homologues of transcriptional regulators, including 13 two-component regulatory systems. However, little is known about the function of most of these regulators, and global regulatory networks have not been identified. Many genes encoding proven or putative transcriptional regulators were differentially expressed in the *covR* mutant strain (Tables 1 and 2). Transcripts of *spy0216* and *spy0735*, which encode two putative transcriptional regulators designated *ralp4* and *ralp3*, respectively, were increased in the *covR* mutant throughout the growth cycle. The encoded proteins have 51% similarity with RofA (SPy0124), a regulator shown to have positive (29) and negative (30) regulatory effects. SPy0216 and SPy0735 also are 51% and 46% similar, respectively, to Nra, a negative regulator of surface adhesins characterized in a serotype M49 strain (9). Hence, SPy0216 and SPy0735 may regulate production of multiple surface molecules or influence the activity of other regulatory networks.

The gene encoding transcriptional regulator Rgg (SPy2042) is adjacent to *speB,* required for *speB* expression (31), and proposed to regulate a GAS response to nutritional stress (11). *Rgg* transcripts were substantially more abundant in the *covR* mutant strain in all growth phases, both in microarrays and TaqMan analysis (Fig. 1). Chaussee and colleagues (32) reported that Rgg negatively influences the expression of the CovR-repressed genes encoding streptodornase (Sda, SPy2043) and a putative extracellular nuclease (SPy1436). CovR has been shown to bind *in vitro* to the promoter region of *sda* (12). Thus, the GAS response to changing environments may be modulated by interactive regulation of gene transcription by CovR and Rgg.

The contiguous genes *spy1587* and *spy1588* encode proteins 46% and 55% similar, respectively, to LytR and LytS, a two-component gene regulatory system affecting cell wall metabolism in *S. aureus* (33). Transcripts of *spy1587* were more abundant in the *covR* mutant strain (Fig. 1 and Table 3, which is published as supporting information on the PNAS web site). Inactivation of *lytRS* in *Streptococcus pneumoniae* decreased virulence in mouse infection models (34). Similarly, insertional inactivation of *lytS* in group B *Streptococcus* decreased virulence for neonatal rats (22).

Transcription of other regulatory loci also was affected by *covR* inactivation. For example, transcripts of the contiguous genes *spy2026* and *spy2027* were more abundant in the *covR* mutant strain (Tables 2 and 3). These genes, previously designated *ihk-irr* (7), encode a putative two-component gene regulatory system. The inferred protein encoded by *spy2027* is 62% similar to ArlR in *S. aureus* (35), and 59% similar to $VanR_B$ in *Enterococcus faecalis* (36), both of which alter peptidoglycan metabolism. Transcripts encoding a putative GntR family transcriptional regulator gene (*spy0715*) (37), and two adjacent genes (*spy0146* and *spy0145*) whose inferred products have 73% similarity to the *Clostridium perfringens* perfringolysin activator (PfoR) (38), and 52% similarity to YjgF family regulators (39), respectively, also were more abundant in the *covR* mutant strain, as was *spy1240* with 60% similarity to the PhoU phosphate transport regulator in *S. pneumoniae* (40) (Table 2). Conversely, five transcripts encoding homologues of transcriptional regulators (SPy0138, SPy0533, SPy1062, SPy1680, and SPy1755) were less abundant in the *covR* mutant strain, primarily in LE phase. The GAS genes controlled by these inferred regulator homologues are unknown.

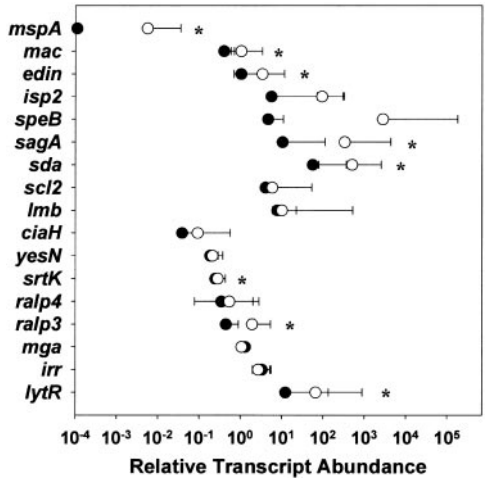

Fig. 3. Relative abundance of GAS transcripts determined by real-time RT-PCR in RNA derived from mouse tissue extracts. Transcripts in GAS-infected tissues were quantified 2.5 days postinoculation in two-step TaqMan assays. Data shown are median transcript levels relative to the *proS* internal reference transcript for each inoculating strain: WT strain MGAS5005 (●); mutant strain JRS950 (○). Error bars represent two standard errors. Significance was determined by using Student's *t* test (*, $P < 0.05$).

Differential Gene Transcription in a Mouse Model of Soft-Tissue Infection. To determine whether CovR-mediated gene expression was altered *in vivo*, we used a mouse s.c. infection model (41) (Table 4, which is published as supporting information on the PNAS web site). Total RNA was prepared directly from GAS-infected tissues recovered 2.5 days postinoculation and transcripts of 17 genes were quantified by TaqMan RT-PCR. Transcripts encoding secreted products SagA, Sda (MF-1), SpeB and Isp2, cell-surface adhesins Lmb and Scl2, and the two-component response regulator LytR were very abundant in GAS-infected soft tissue, detected at approximately 10-fold higher levels than control gene *proS* (Fig. 3). Of these seven genes, association with virulence in murine soft-tissue infection models has been reported only for *speB* and *sagA* (41, 42). Relative levels of *mspA, mac*, *edin, sagA*, *sda, srtK, ralp3*, and *lytR* transcripts were significantly higher ($P < 0.05$, *t* test; Fig. 3) in RNA derived from *covR* mutant-infected mice relative to WT-infected mice. This finding indicates that these genes are regulated by CovR in infected hosts as well as *in vitro* (Table 5, which is published as supporting information on the PNAS web site). This also appeared to be likely for most other genes (15/17)

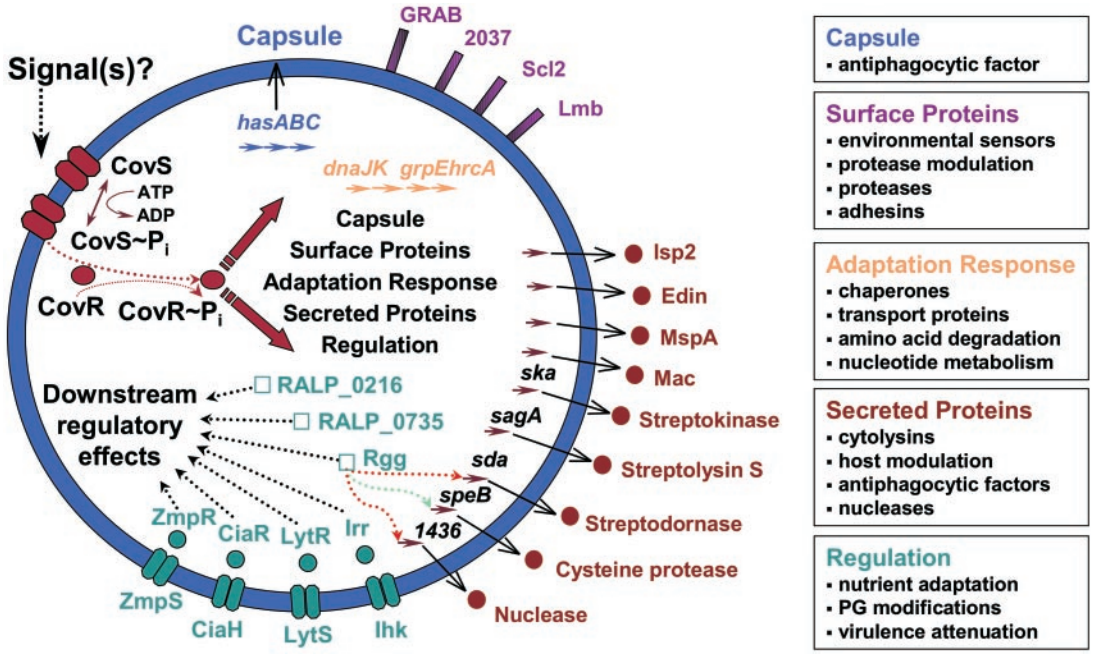

Fig. 4. GAS CovR gene regulation network delineated by microarray expression analysis. CovRS respond to unknown environmental signals and modulate expression of several broad categories of GAS genes involved in growth and adaptation by a phosphorelay mechanism. CovR-regulated gene products influence host–pathogen interactions, including capsular polysaccharide (capsule), cell proteins anchored via LPXTG motifs (surface proteins), cytosolic and membrane proteins involved in environmental adaptation (adaptation response), and extracellular proteins containing secretion signals (secreted proteins). Several two-component systems (ovals) and other transcriptional regulators (squares) are also CovR-regulated, but their downstream regulatory consequences are unknown. Red lines, down-regulation; green lines, up-regulation. PG, peptidoglycan; ●, secreted proteins; surface solid rectangles, secreted proteins with LPXTG motifs. Numbers denote SPy numbers assigned for serotype M1 GAS strain SF370 ORFs.

analyzed, although the transcript level differences in mice did not reach statistical significance (Fig. 3). Our findings concur with immunologic data indicating that secreted antigens Mac and Sda are made during the course of human infection (18). Importantly, data obtained for regulator transcripts *ciaH* (*spy1236*), *yesN* (*spy1061*), and *irr* (*spy2027*) differed from the *in vitro* expression data, suggesting that these genes are influenced *in vivo* by an additional regulatory mechanism(s) that is not required *in vitro*. This observation stresses the importance of comparing data derived from *in vitro* studies and relevant animal models or human infections.

Discussion

Our analysis indicates that CovR likely directly or indirectly influences transcription of 15% (n = 271) of GAS genes, dispersed throughout the genome, including genes involved in diverse cellular activities such as stress adaptation, gene regulation, and pathogenesis. CovR-repressed genes include many that encode surface and secreted products expected to participate in host–pathogen interactions (Fig. 4). Although it is likely that CovR directly regulates some of the genes we identified, many of the transcription differences between the WT and mutant strains probably are caused by indirect actions of CovR mediated through collateral regulatory networks. Three observations are consistent with this idea. First, the complex temporal pattern of differential gene expression makes it unlikely that CovR directly mediates all observed effects. Second, in addition to *covR*, transcription of genes encoding five of the 12 other putative two-component response regulators was altered substantially. Third, in the aggregate, 24 homologues of other transcriptional regulatory genes had altered expression.

Inasmuch as CovR affects transcription of such a large number of genes, and some of these genes are themselves gene regulators, it is likely that CovR is part of a regulatory network(s). Consistent with this idea, we find some genes to be CovR regulated under some but not all growth conditions (e.g., mouse vs. *in vitro*). To unravel gene regulatory interactions, promoters controlled directly by CovR need to be distinguished from those controlled indirectly, a process requiring further analyses. Analysis *in silico* of the promoter regions of 14 of the most differentially expressed genes did not reveal a conserved nucleotide motif (details of the analysis are published as supporting information on the PNAS web site).

Correlation of DNA Microarray and TaqMan Data. The majority of genes identified as differentially expressed by microarray analysis were confirmed to have an altered level of transcription with TaqMan assays (Fig. 2). Most differentially expressed genes had higher expression differences by TaqMan assays, probably reflecting differences in the relative sensitivity and specificity of the two methods. Microarrays comprised of spotted PCR products are subject to binding saturation by the finite amounts of fixed DNA on the slide. TaqMan probes are not limiting, hybridize in solution, and can exhibit a linear relationship over 4 orders of magnitude. Consequently, TaqMan RT-PCR is more efficient than microarrays in detecting low-abundance transcripts (43).

CovR Inactivation and Survival *in Vivo*. The mutant and parental strains had identical growth rates and growth yields *in vitro* (Fig. 1*A*), indicating that disruption of CovR function confers no apparent advantage or disadvantage to GAS grown under this condition. However, to survive in the host GAS must successfully respond to changes in a variety of environmental conditions. The CovRS gene regulatory system provides a sensitive molecular mechanism to mediate rapid changes in expression of gene products that interact with the host. The diverse functional categories of differentially expressed genes identified by our

analysis suggest that CovRS-influenced adaptive responses are likely to promote survival in the host. Data consistent with this idea have been generated from experimental infection of mice (14). Moreover, some GAS strains cultured from infected humans have frameshift mutations in *covRS* that inactivate signal transduction by this system (14). Although the extent to which these processes moderate infection severity and outcome in humans is unknown, our results indicate that disruption of CovR repressor activity *in vivo* significantly alters production of many proven and putative virulence factors.

GAS vaccine candidates and known virulence factors have been identified primarily with *in vitro* methods. However, host environmental factors that influence bacterial gene regulation and/or function at infection sites or in systemic circulation are complex and difficult to simulate. Expression transcript profiling, coupled with TaqMan assays, will undoubtedly provide insight into complex gene regulatory circuits operative *in vivo* and assist pathogenesis research.

Concluding Comment. GAS strains vary considerably in gene content, allelic diversity, and gene expression levels (44) and hence, the extent to which the findings are broadly applicable is unknown. We analyzed RNA harvested at three time points after growth at 37°C in rich medium, and at one time point during soft-tissue infection. Analysis of organisms cultured in other conditions, or recovered from ill humans or animals with other experimental GAS infections, may yield different results. Our studies indicate that a complex regulatory network is operative in serotype M1 GAS, organisms responsible for an inordinate amount of human morbidity. Additional gene targets for GAS pathogenesis and *in vivo* gene regulation research have been revealed. Importantly, our results demonstrate that transcription of multiple GAS genes can be analyzed in complex clinical samples, and that CovR is a principal gene regulator operative in model soft-tissue infection. Analogous *in vivo* studies with other virulent microbes or other infection models are possible. Studies of this type will aid our understanding of infectious disease pathogenesis and assist development of new therapeutics, including vaccines.

We thank N. Hoe for critical reading of the manuscript, R. Larson and G. Sylva for technical assistance, and S. Lukomski, B. Lei, and members of the laboratories of S. Falkow, V. Kapur, and D. Relman for helpful discussions. This work was supported in part by Public Health Service Grants AI20723 and T32AI07470.

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