Dissection of the Molecular Basis for Hypervirulence of an In Vivo–Selected Phenotype of the Widely Disseminated M1T1 Strain of Group A Streptococcus Bacteria

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Group A streptococci (GAS) may engage different sets of virulence strategies, depending on the site of infection and host context. We previously isolated 2 phenotypic variants of a globally disseminated M1T1 GAS clone: a virulent wild-type (WT) strain, characterized by a SpeB+/SpeA−/H11002−/Sda1low phenotype, and a hypervirulent animal-passaged (AP) strain, better adapted to survive in vivo, with a SpeB−/SpeA+/Sda1high phenotype. This AP strain arises in vivo due to the selection of bacteria with mutations in covS, the sensor part of a key 2-component regulatory system, CovR/S. To determine whether covS mutations explain the hypervirulence of the AP strain, we deleted covS from WT bacteria (∆covS) and were able to simulate the hypervirulence and gene expression phenotype of naturally selected AP bacteria. Correction of the covS mutation in AP bacteria reverted them back to the WT phenotype. Our data confirm that covS plays a direct role in regulating GAS virulence.

The pathogenesis of group A Streptococcus (GAS) infections reflects the complex interplay between bacterial and host factors. GAS diseases vary from uncomplicated pharyngitis to life-threatening streptococcal toxic shock syndrome and necrotizing fasciitis. Multiple virulence factors play important roles in distinct host niches and different stages of GAS infections [1, 2]. Depending on the site of infection and host context, GAS may engage different sets of virulence programs. For example, the hyaluronic acid capsule, surface-bound M and M-like proteins, fibronectin-, collagen-, and plasminogen-binding proteins, SpeB, immunoglobulin-binding, degrading, and inactivating proteins, C5a-peptidase, and α2-macroglobulin-binding protein are all used in the primary stages of infection, where they promote bacterial adherence, colonization, resistance to phagocytosis, invasion of host cells, and evasion of host defenses [2–4]. Secreted proteins, including streptococcal superantigens, cytotoxins (streptolysin O and streptolysin S), and cell wall–associated peptidoglycans and lipoteichoic acid, elicit inflammatory responses that divert host defenses, allowing bacterial colonization of specific host niches [1, 5–7]. Furthermore, secreted deoxyribonucleases (hereafter, DNases) allow the bacteria to escape neutrophil killing [8, 9].

Under host pressure, the composition of the bacterial community can drastically change, revealing phenotypes that differ from the original inoculum [9–11].

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Such phenotypic switching has been documented for the globally disseminated M1T1 strain and may be a part of the bacterial strategy to evade host defense mechanisms [12]. Analysis of the in vivo–manifested phenotypes uncovered molecular events associated with the emergence of a virulent M1T1 clonal strain that differs markedly from its ancestral M1T1 SF370 strain in prevalence, dissemination, and virulence [12–17]. This clonal M1T1 strain emerged in the 1980s, coinciding with the resurgence of streptococcal toxic shock syndrome and necrotizing fasciitis [12]. Its virulence and prevalence have been partially attributed to the acquisition of 2 prophages, which introduced the speA and sda1 genes, encoding the superantigen SpeA and the highly potent DNase Sda1, respectively [14, 15].

In analyzing large numbers of clinical isolates, we identified 2 phenotypic variants of the emergent M1T1 strain that differed significantly in their expression of the virulence factors SpeB, SpeA, and Sda1. Using our chamber model of localized GAS infection [11], we separated these 2 phenotypes. The wild-type (WT) phenotype is characterized by high expression of SpeB but low expression of SpeA and Sda1 (SpeB+/SpeA−/Sda1low), and the animal-passaged (AP) phenotype expresses no SpeB but high levels of SpeA and Sda1 (SpeB−/SpeA+/Sda1high) [10, 11]. These variants differ considerably in their invasive potential, virulence, and ability to thrive in vivo [9, 18, 19]. Interestingly, all bacteria exhibiting the AP phenotype have mutations in covS [9, 12, 19], the sensor part of a key 2-component regulatory system CovR/S that regulates expression of many streptococcal genes [20–23].

We undertook this study to determine whether the hyper-virulent phenotype of the AP M1T1 bacteria is directly related to the covS mutation, or whether other factors contribute more significantly to its invasive properties and hypervirulence.

**METHODS**

**Generation of in-frame covS allelic exchange knockout and reverse-complemented strains.** We used the parental WT and AP phenotypic variants of 2 representative M1T1 GAS clinical isolates 5448 and 6050 [6]. We amplified a 2253 base pair (bp) GAS chromosomal DNA fragment containing covS, along with and the animal-passaged (AP) phenotype expresses no SpeB but high levels of SpeA and Sda1 (SpeB−/SpeA+/Sda1high) [10, 11]. These variants differ considerably in their invasive potential, virulence, and ability to thrive in vivo [9, 18, 19]. Interestingly, all bacteria exhibiting the AP phenotype have mutations in covS [9, 12, 19], the sensor part of a key 2-component regulatory system CovR/S that regulates expression of many streptococcal genes [20–23].

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Figure 1. SpeB+/SpeA+/Sda1high AP bacteria are more virulent than SpeB−/SpeA−/Sda1low WT bacteria in CBA mice. Kaplan-Meier Survival curves of CBA/J mice infected intravenously with 2 × 10⁶ CFU of the WT bacteria or the in vivo–derived AP bacteria of 2 M1T1 group A Streptococcus (GAS) isolates, 5448 (A) and 6050 (B). Mouse survival was recorded on daily basis. Statistical significance in survival curves was determined by the log-rank test. (C) Bacterial loads in blood following infection: At 24 h after infection, 20 μL blood was drawn aseptically from the tail vein of mice infected with the WT or AP bacteria of 5448 or 6050 GAS, and the bacterial loads were determined by plating a 100-fold dilution of the blood on the blood agar plates. The plates were incubated at 37°C for 24 h, and β-hemolytic colonies were counted. Differences between bacterial counts in blood were analyzed by 2-tailed Student t test. (D) Loss in body weights following infection. Infected mice were weighed daily for the entire observation period or until they died as a result of the infection. The weight loss is presented as the percentage of mice initial weight. Differences between weight loss were analyzed with 2-tailed Student t test. The results presented are representative of 2 or 3 experiments.
flanking upstream and downstream sequences. The polymerase chain reaction (PCR) product was T-A cloned in pCR2.1 sequence (Invitrogen) to give p covS-TV, which served as a template for an inverse PCR reaction using (1) a reverse primer immediately upstream of the start codon and (2) a forward primer immediately after the stop codon of covS. Primers were designed with 25 bp 5'-extensions corresponding to the start and end of the chloramphenicol acetyltransferase gene (\textit{cat}). The linearized PCR product, containing an in-frame deletion of \textit{covS}, was used to transform \textit{Escherichia coli} Top10 (Invitrogen), together with an \textasciitilde{}650 bp PCR amplicon of the complete \textit{cat} gene from pACYC184. We identified in vivo recombination events by selecting for Km\textsuperscript{R} + Cm\textsuperscript{R} colonies and verified them with PCR and restriction analyses. The mutated \textit{covS\Delta cat} gene and its flanking DNA was subcloned as a \textit{BamH}I/\textit{XhoI} fragment into the temperature-sensitive Em\textsuperscript{R} vector pHY304 to produce knockout vector p covS\textit{\Delta cat}-KO. Competent bacteria were transformed with p CovS\textit{\Delta cat}-KO, and single recombination events were identified at 37\textdegree C under Em selection [24]. Selection was relaxed by serial passages at 30\textdegree C without antibiotics, and double-crossover events were identified as Cm\textsuperscript{R}, but Em\textsuperscript{R} 5448 mutants exhibiting precise in-frame allelic ex-
change of covS with cat in the GAS chromosome were verified by PCR. The confirmed mutant was designated 5448 ΔCovS. We also used reverse complementation to replace the mutated chromosomal covS in the AP strain with native covS (AP-RCnatcovS), as described elsewhere [9].

Other procedures. Western blot analyses and measurement of secreted DNAse activity were performed, as described elsewhere [6, 18].

Infection studies. Female CBA/J mice (6–8 weeks old) were obtained from Jackson Laboratories and housed under standard Institutional Animal Care and Use Committee approved conditions. Mice were infected intravenously with (2–3) × 10⁶ colony-forming units (CFU) of bacteria in 250 μL phosphate-buffered saline; 24 h later, 20 μL of blood was drawn from the tail vein to assess bacteremia [25]. To assess infection severity, we monitored daily weight loss.

Statistical analysis. Statistical differences in weight loss and blood CFU were analyzed with the Student t test (2 tailed). Kaplan-Meier survival curves were generated to compare mice survival, and the statistical significance was calculated with the log-rank test. A value of P < .05 was considered to indicate a statistically significant difference.

Gene expression profiling. For comprehensive transcriptome analysis, we printed oligonucleotide spotted glass microarrays at our Molecular Resource Center, representing all the open reading frames of the M1T1 GAS, using oligo sets designed and provided by Doctors J. Scott and K. McIver, with additional open reading frames (mainly phage encoded) from

Figure 4. 5448ΔCovS is as virulent as animal-passaged (AP) bacteria, and both are significantly more virulent than wild-type (WT) bacteria. (A) Kaplan-Meier Survival curves of survival of CBA/J mice infected intravenously with (2–3) × 10⁶ CFU of WT, AP, and 5448ΔCovS bacteria (n = 10). Mouse survival was recorded on a daily basis. Differences between survival curves was determined by the log-rank test. (B) Bacterial loads in blood following infection. At 24 h after infection, 20 μL of blood was drawn aseptically from the tail vein of infected mice, and the bacterial loads were determined by plating a 100-fold dilution of the blood on the blood agar plates. The plates were incubated at 37°C for 24 h, and β-hemolytic colonies were counted. Differences between bacterial counts in blood were analyzed by 2-tailed Student t test. (C) Loss in body weight following infection. Infected mice were weighed daily for the entire observation period or until they died as a result of the infection. The weight loss is presented as the percentage of their initial weight. Differences between weight loss were analyzed by 2-tailed Student t test. The results are representative of 2 or 3 experiments. (D) Western blot analyses of secreted SpeB, SpeA, and Sda1 in supernatants of WT (lane 1), AP (lane 2), 5448ΔCovS (lane 3), or AP-RCnat covS (lane 4) bacteria of M1T1 GAS.
other strains. Each open reading frame was spotted in duplicate. RNA was extracted from stationary phase (18 h) bacteria and freed of contaminating DNA by Turbo DNase treatment (Ambion) [6, 18]. Purified RNA quality was assessed by bioanalyzer (Agilent Technologies) and reverse transcribed into complementary DNA using SuperScriptII reverse transcriptase (Invitrogen) and labeled with Alexa Fluor 546/647 by using a 3DNA Array 900 MPX kit (Genisphere). RNA was isolated from 3 biological replicates, each analyzed on at least 3 different microarrays (3 technical replicates), resulting in >12 data points for each gene per experimental conditions.

We scanned the microarrays using a GenePix 4000B scanner and performed primary analyses using GenePixPro software (version 4.0; Axon Instruments). The analyses included spot finding, alignment and adjustment, fluorescence normalization, flagging of poorly hybridized spots, and background subtraction. We performed subsequent analyses by using GeneSpring GX software (version 7.3; Agilent Technologies). To compare gene expression profiles of any 2 groups, we used GeneSpring’s parametric test and assumed unequal variance. Microarray data validation was conducted using reverse-transcription PCR (RT-PCR) assays, as described elsewhere [6].

RESULTS

Stark differences in virulence of SpeB+/SpeA−/Sda1low (WT) and SpeB−/SpeA+/Sda1high (AP) bacteria. We infected susceptible CBA/J mice [26] with 2 × 10^6 CFU of WT or AP GAS bacteria. To rule out isolate-specific effects, we used 2 MIT1 GAS isolates, 5448 and 6050, and found them behaving similarly in vitro and in vivo. Mice infected with 5448AP or 6050AP bacteria started to die as a result of the infection by day 3, and 8 (89%) of the 9 mice infected with the 5448AP bacteria and 7 (88%) of the 8 mice infected with 6050AP bacteria had died by day 7. In contrast, 90%–100% of mice infected with 5448WT or 6050WT survived until the end of the observation period (Figure 1).

Increased virulence of AP bacteria was associated with their superior ability to survive and multiply in blood. Mice infected with WT bacteria cleared the infection significantly faster than those infected with AP bacteria (Figure 1C). Bacterial loads in the blood of mice infected with AP bacteria were 3 log higher than those infected with WT bacteria (4.3 ± 0.4 log CFU/mL vs 1.4 ± 1.5; P < .001). Furthermore, the percentage of body weight loss following infection with AP bacteria (20.6% ± 5.6%) was 5-fold higher than those infected with WT bacteria (4.2% ± 7.2%; P < .001) (Figure 1D).

Molecular basis for hypervirulence of AP bacteria. Both AP strains showed higher expression of Sda1 and SpeA and absence of SpeB compared with their WT counterparts, and both have a mutation in covS. We had reported that covS in 5448AP bacteria has a 1-base insertion at its 3′ end [9], resulting in premature termination at amino acid 300 from the start codon of the predicted 500-amino-acid protein. Here, we found that the 6050AP bacteria also have a 5-nucleotide deletion (GAAAA) at nucleotide 1214 from the 3′ end of covS, resulting in premature termination at amino acid 417 from the start codon (Figure 2). Both mutations are expected to truncate the histidine kinase domain of CovS. Also, both are associated with the hypervirulent phenotype of 5448AP and 6050AP bacteria that are phenotypically similar to invasive transcriptome profile

Figure 5. Correction of the covS mutation in AP bacteria by reverse complementation reverts them to the WT phenotype. (A) Kaplan-Meier survival curves of CBA/J mice infected intravenously with 2 × 10^6 CFU of the WT phenotype, the AP phenotype, 5448ΔcovS, or AP-RCnatcovS (n = 8). Mouse survival was recorded on a daily basis. Differences between survival curves were determined by the log-rank test. (B) Bacterial loads in blood following infection. At 24 h after infection, 20 μL blood was drawn aseptically from the tail vein of infected mice, and the bacterial loads were determined by plating a 100-fold dilution of the blood on the blood agar plates. The plates were incubated at 37°C for 24 h, and β-hemolytic colonies were counted. Differences between bacterial counts in blood were analyzed by 2-tailed Student t test.
Figure 6. (A) Gene expression profiling of the wild-type (WT), animal-passaged (AP), and ΔCovS bacteria. A heat map of clustered normalized expression values of differentially expressed genes between WT, AP, and ΔCovS bacteria (average of all replicates, n = 10). Differentially expressed genes were defined as those with change in expression at >2-fold difference and P value cutoff of .05 using Volcano Plot analysis of the GeneSpring GX software (version 7.3). (B) Real-time polymerase chain reaction (PCR) validation of microarray expression results. Complementary DNA was synthesized from total RNA of WT, AP, and ΔCovS bacteria, and expression of gene-specific transcripts was quantified by real-time PCR as explained in the Methods section. The gene-specific transcripts were normalized to the housekeeping gene gyrA, and fold change in expression of a particular gene was calculated using 2^(-ΔΔCt) method.

[19] of MGAS5005 that also has a truncated CovS at amino acid 454 and MGAS2221, with a frame-shift mutation resulting in truncation of the protein at amino acid 202 (Figure 2). To directly address whether mutations in covS account for the hypervirulence of various AP strains of M1T1 bacteria, we generated an allelic exchange mutant of covS in WT 5448 and tested its virulence.

Initial characterization of the 5448ΔCovS mutant showed an expression profile of SpeB, SpeA, and Sda1 mimicking the naturally selected M1T1 AP bacteria (SpeB+/SpeA+/Sda1high) (Figure 3). Importantly, the 5448ΔCovS mutant was as virulent as the AP bacteria. Mice (n = 10) infected with (2–3) × 10^6 CFU of 5448ΔCovS mutant or 5448 AP bacteria showed 100% and 90% mortality, respectively, but most died by days 3 or 4. In contrast, none of the mice infected with the same dose of WT 5448 bacteria died within the first 4 days; afterward, gradual mortality was seen, with 50% of the mice surviving to the end of the observation period. In conclusion, the virulence of 5448ΔCovS bacteria was similar to that of 5448 WT bacteria (P< .001 and P = .005, respectively; Figure 4A). Similarly, bacterial loads in blood of mice infected with 5448ΔCovS or 5448AP bacteria were 3 log higher than those in blood of mice infected with WT bacteria (4.5 ± 0.3 log CFU/mL [ΔCovS] and 4.6 ± 0.4 [AP] vs 1.0 ± 1.2 [WT], P< .001; Figure 4B). The increased virulence of both bacteria types was also marked by greater weight loss (Figure 4C).

Correction of the covS mutation in AP bacteria by reverse complementation reverts them to the WT phenotype. The above studies suggested that the in vivo acquired mutations in covS contributed substantially to the increased virulence of AP bacteria. To challenge our conclusion, we corrected the mutated covS in 5448AP bacteria (5448AP-RCnatcovS) and compared its virulence to that of WT and 5448AP bacteria.

Correction of the covS mutation in 5448AP bacteria restored the expression profile of SpeB, SpeA, and Sda1 back to WT bacteria (SpeB+/SpeA+/Sda1low). Importantly, the corrected strain behaved like WT bacteria in vivo, with significantly attenuated virulence compared with 5448ΔcovS and 5448AP bacteria. Following infection of mice with (1–2) × 10^6 CFU, 8
Figure 6. Continued
Effects of covS deletion/mutations on global gene expression in M1T1 GAS. To study whether the increased virulence of the AP and ΔCovS bacteria can solely be attributed to differences in their expression of SpeB, SpeA, and Sda1, or whether other genes can also contribute considerably to hypervirulent phenotype, we conducted comprehensive transcriptome comparisons between 5448WT versus AP/ΔCovS bacteria. We found 96 genes were differentially expressed between 5448WT versus AP bacteria, and 102 genes were differentially expressed between WT versus ΔCovS bacteria. Of these, only 25 genes were shared between both AP and ΔCovS bacteria (Figure 6A) and included several genes implicated in GAS virulence. Although genes encoding for M protein, streptococcal inhibitor of complement (SIC), streptolysin O, hyaluronic acid capsule, collagen-like surface protein, streptokinase, C5a peptidase, Sda1, and superantigens SpeA and SpeJ were up-regulated in both AP and ΔCovS strains, genes within the SpeB operon and CAMP (Christie Atkins Munch-Peterson) factor were significantly down-regulated (Table 1). Additionally, glycosyl hydrolase (Spy1304) and maltodextrin utilization protein MalA (Spy2318) genes involved in maltose and maltodextrin utilization were up-regulated in both AP and ΔCovS bacteria (Table 1). This was further confirmed by quantitative RT-PCR analyses (Figure 6B). Although the expression patterns of most genes identified through microarray analyses matched quantitative RT-PCR analyses, few were discordant. Of particular importance were capsule synthesis genes. Although we observed 100-fold increase in expression of hasA, hasB, and hasC in both AP and ΔCovS bacteria by using quantitative RT-PCR (Figure 6B), none except hasB could be identified through microarray analyses (Figure 6A; Table 1). Similarly, a transcriptional regulator

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<th>Fold Change ΔCovS vs WT</th>
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NOTE. NADGH, nicotinic adenine dinucleotide glycohydrolase; SIC, streptococcal inhibitor of complement; SLO, streptolysin O.
(Spy2099) belonging to GntR family showed up-regulation in both AP and ΔCovS bacteria at microarray analysis but not at quantitative RT-PCR analysis. These discrepancies may be due to differences in binding efficiencies of the probes used in the 2 platforms. As predicted, gene expression profile of AP-RCnatCovS bacteria mirrored that of WT bacteria.

Despite apparent similarities between the transcriptome profiles of ΔCovS and AP bacteria versus WT bacteria, few genes were affected differently in AP and ΔCovS bacteria. For example, the pilus-associated sortase (Spy0129) and pilus-backbone proteins as well as streptococcal 2-component system response regulator ciaR (Spy1237) and its putative sensor histidine kinase ciaH (Spy 1238) were significantly up-regulated only in ΔCovS bacteria (Figure 6B). Furthermore, several other genes were affected exclusively in either the AP or ΔCovS bacteria (see the supplement lists in the Appendix, which appears only in the electronic edition of the Journal), but to our knowledge most of these have not yet been implicated in any important biological function.

The above results demonstrate that mutations in the histidine kinase domain of covS play a major role in conferring hypervirulence on AP bacteria. Even though different mutations may affect different sets of genes, all share the SpeB/SpeA+/Sda1high phenotype. This increased virulence is brought about through coordinated changes in expression of certain putative and proven virulence factors and regulators, as well as genes involved in amino acid and maltodextrin utilization.

**DISCUSSION**

The interactions between GAS and its host reflect attempts by the bacteria to survive within a host niche so that they can infect another host and perpetuate themselves. As seen in this study, these attempts can lead to the restructuring of the bacterial community in a way that allows its fittest members to survive. Employing a mouse model of GAS-sepsis and toxic shock, we found that M1T1 AP bacteria, which are barely detected in the WT bacterial community, take over to become the bacterial majority in vivo, presumably because they have a selective survival advantage over WT bacteria [11, 12]. Sumby et al [19] reported a similar phenomenon that the invasive strain MGAS5005 and animal passaged MGAS2221 also acquire mutations in covS (Figure 2) [27]. Accordingly, we had hypothesized that covS mutations, which mark all the in vivo-selected AP bacteria from different M1T1 strains, may increase their virulence [12]. However, the reason behind the hypervirulence of these CovS mutants was not entirely clear because a direct demonstration that covS deletion would generate the same hypervirulent phenotype was lacking. Here, we provide direct evidence that an intact covS is responsible for the attenuated GAS virulence, inasmuch as deleting covS converted the WT bacteria to a hypervirulent phenotype and correcting covS mutations in AP bacteria reverted them back to their attenuated WT phenotype.

Our molecular studies suggest a direct link between covS mutations and deletions and profound changes in M1T1 GAS virulome. Hypervirulent variants of M1T1 bacteria, including 5448, 6050, MGAS2221, or MGAS5005, have different mutations in covS [19], all clustered in its histidine kinase domain (Figure 2), but the underlying mechanism of this phenomenon is not entirely clear. The data presented here provide some insight into this phenomenon.

We dissected phenotypic differences between WT, AP, and ΔCovS bacteria and found complete down-regulation of SpeB, and significant up-regulation of SpeA and Sda1 in both natural and engineered CovS mutants. SpeB down-regulation in AP bacteria preserves important virulence factors, including several streptococcal superantigens [10, 28], and increases GAS invasiveness and virulence [8, 9]. The clinical relevance of this phenomenon was demonstrated previously, when we found that M1T1 isolates from patients with severe GAS sepsis and streptococcal toxic shock syndrome were more likely to exhibit the AP phenotype, whereas those from nonsevere bacteremia cases were more likely to exhibit the WT phenotype [18]. Present gene profiling studies revealed that several additional key virulence factors were not only spared in the covS (SpeB+)) mutants, but that their expression was significantly up-regulated in both AP and ΔCovS bacteria. These included anti-phagocytic M protein, anti-chemotactic factor C5a peptidase, streptolysin O cytolysin, adhesion collagen-like surface protein, SIC, the superantigen SmeZ, and cytotoxic Streptococcus pyogenes NAD-glycohydrolase. Up-regulating and sparing these factors is expected to increase bacterial virulence.

Additional major changes effected by CovS mutations/deletion included increased expression of hasABC operon, ska, sagA, and streptococcal interleukin 8 protease (ScpC or SpyCEP). Whereas the induction of hasABC operon increases capsule synthesis and is expected to increase virulence, increased ska expression is not expected to have an effect in regular animal models of GAS sepsis inasmuch as GAS Ska is specific for only human plasminogen [29]. Consistent with findings in previous studies [30–32], we found that sagA expression was up-regulated in AP and ΔCovS mutants. By contrast, Sumby et al [19] showed reduced sagA transcripts in covS mutants. Induction of SagA, which encodes streptolysin S, a toxin that damages membranes of eukaryotic cells and subcellular organelles, is expected to increase virulence in our animal model [33, 34]. Up-regulation of ScpC, which allows GAS to resist neutrophil killing in both AP and ΔCovS mutants (and which is also observed by Grylllos et al [35]), likely contributes to hypervirulence. It was directly shown by Zinkernagel et al [36] that the ScpC knockout mutant had attenuated virulence in murine infection models.

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Our data also indicate that distinct mutations in covS or its deletion may have different effects on expression of certain genes. Of the ≥100 differentially expressed genes between either AP or ΔcovS bacteria and WT bacteria, only 25 were shared between them. We believe that the AP strain may have additional mutations not found in the covS deletion mutant that might have been enriched during the in vivo passage. For example, genes encoding the pilus-associated sortase (SPY0129) and pilus-backbone proteins—which are located within the fibronectin, collagen binding locus and have gained much recent interest due to their role in adherence to eukaryotic surfaces and GAS virulence [37, 38]—were significantly up-regulated only in ΔcovS but not in AP bacteria (Figure 6B). Also, we did not observe increased expression of the immunoglobulin G protease IdeS/Mac in CovS mutants, as seen by Grylls et al [35]. Finally, whereas genes involved in maltodextrin utilization were similarly up-regulated in AP and ΔcovS bacteria, these genes are not known to be directly regulated by CovR/S. However, they are believed to contribute to GAS oropharynx infections [39–41].

Although the CovR/CovS system is one of the best-studied 2-component regulatory systems of GAS because it directly or indirectly regulates many genes [42], much remains to be learned about this system. Phosphorylated CovR is believed to negatively regulate many GAS genes, including speB, hasA, ska, speF, and sagA [31, 43, 44]. The mechanism by which CovR suppresses expression of these genes is not clear. Similarly, the mechanism by which different covS mutations, mostly clustered within a histidine kinase domain, alters CovR-mediated gene regulation and affects bacterial virulence is also unclear. Originally, CovS, a histidine kinase domain, was thought to phosphorylate CovR at D53, but Dalton and Scott [45] showed that CovR can be phosphorylated at D53 by acetyl phosphate in the absence of CovS. Some histidine kinase domains are bifunctional, catalyzing both the phosphorylation and dephosphorylation of their cognate response regulators, where the input stimulus can regulate either their kinase or phosphatase activity [45, 46].

Because the CovR/S system affects genes belonging to multiple functional categories, the relative contribution of each category or individual virulence factors to GAS virulence remains to be elucidated. Although many of the virulence factors controlled by CovR/S have been shown independently to play an important role in GAS pathogenesis, their interactive (additive/synergistic/antagonistic) effects need to be investigated and the underlying mechanism need to be better understood [45]. It is possible that intact CovS dephosphorylates CovR under certain conditions, thereby releasing it from its cognate promoters and restoring expression of repressed genes, like SpeB, whereas mutated CovS is unable to fulfill this function, resulting in permanent SpeB repression in AP and CovS mutant bacteria. This theory, however, does not explain why hasA, ska, and sagA genes—all believed to be negatively regulated by Covr-P—were up-regulated instead of repressed in mutant covS bacteria. Clearly, detailed structure-function studies and in-depth analyses of the ways that different stimuli can affect the way CovS modulates CovR-mediated gene regulation are needed to reveal how various mutations in covS may differentially affect expression of CovR regulated genes and help us to better understand this highly complex system.

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