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Regulatory gene mutation: a driving force behind group A *Streptococcus* strain- and serotype-specific variation

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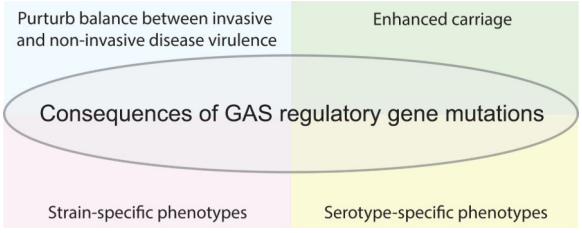
Abstract

Data from multiple bacterial pathogens are consistent with regulator-encoding genes having higher mutation frequencies than the genome average. Such mutations drive both strain- and type- (e.g. serotype, haplotype) specific phenotypic heterogeneity, and may challenge public health due to the potential of variants to circumvent established treatment and/or preventative regimes. Here, using the human bacterial pathogen the group A *Streptococcus* (GAS; *S. pyogenes*) as a model organism, we review the types and regulatory-, phenotypic-, and disease-specific consequences of naturally occurring regulatory gene mutations. Strain-specific regulator mutations that will be discussed include examples that transform isolates into hyper-invasive forms by enhancing expression of immunomodulatory virulence factors, and examples that promote asymptomatic carriage of the organism. The discussion of serotype-specific regulator mutations focuses on serotype M3 GAS isolates, and how the identified rewiring of regulatory networks in this serotype may be contributing to a decades old epidemiological association of M3 isolates with particularly severe invasive infections. We conclude that mutation plays an outsized role in GAS pathogenesis and has clinical relevance. Given the phenotypic variability associated with regulatory gene mutations, the rapid examination of these genes in infecting isolates may inform with respect to potential patient complications and treatment options.

Graphical abstract



Abbreviated summary

The selection of mutations within regulator-encoding genes is a major driver of bacterial strain- and type-specific phenotypic heterogeneity. Here, we review the types and regulatory-, phenotypic-, and disease-specific consequences of naturally occurring regulatory gene mutations

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in the human bacterial pathogen the group A *Streptococcus*. We conclude that mutation plays an outsized role in group A *Streptococcus* pathogenesis and has clinical relevance.

Introduction

Bacterial pathogens commonly show intraspecies variation which has led to the creation of classification systems by which isolates can be distinguished (e.g. different clones, pulsed-field types, or serotypes). Importantly, at least in some instances it has been discovered that isolates from different classification groups have distinct virulence characteristics. For example, *Staphylococcus aureus* isolates from pulsed-field type USA300 show enhanced virulence relative to isolates from pulsed-field type USA200, and in part this is explained by differences in the Agr regulatory system which is a major regulator of virulence factor expression (Cheung *et al.*, 2011, Uhlemann *et al.*, 2014). Similarly, certain *Salmonella enterica* serotypes have the potential to become hyper-infectious following animal passage, although the molecular basis of this is unknown (Heithoff *et al.*, 2012). Further, the human-specific pathogen group A *Streptococcus* (GAS, *Streptococcus pyogenes*) shows strain- and serotype-specific disease associations (Mitchell, 1962, Walker *et al.*, 2007). Despite the public health challenges associated with variant emergence (Nicol & Wilkinson, 2008, Croucher *et al.*, 2011), in most instances there is a dearth of information regarding the molecular mechanisms that drive this variation. Here, to summarize how the mutation-induced rewiring of regulatory networks can influence pathogen phenotypic heterogeneity, we will review how it drives strain- and serotype-specific phenotypic variation in GAS.

GAS cause human diseases that range from self-limiting pharyngitis, of which there are >600 million cases annually (Ralph & Carapetis, 2013), to the severely invasive necrotizing fasciitis, which has a mortality rate of between 25 and 50% (Olsen & Musser, 2010). The ability of this pathogen to cause such disease diversity is a consequence of its ability to express different virulence factor profiles, selected from dozens of secreted and cell-wall anchored proteins, in response to internal and external environmental cues (Hondorp & McIver, 2007, Shelburne *et al.*, 2008). To this end, GAS utilize a combination of two-component regulatory systems, stand-alone transcription factors, and small non-coding regulatory RNAs (sRNAs) (Ribardo *et al.*, 2004, McIver, 2009, Perez *et al.*, 2009, Vega *et al.*, 2016).

GAS strains are divided into serotypes based upon the sequence of the 5’ end of the *emm* gene, which encodes the classical GAS virulence factor the M protein (Cunningham, 2014). Importantly, epidemiological analyses spreading back more than five decades have identified non-random associations between certain serotypes and particular disease manifestations. For example, serotype M3 GAS isolates are associated with causing particularly severe invasive infections with a high mortality rate, while serotype M18 strains are associated with outbreaks of the post-GAS-infection sequela acute rheumatic fever (Mitchell, 1962, Beres *et al.*, 2006). While the molecular mechanisms driving GAS-serotype disease-phenotype associations have yet to be fully elucidated, in some instances the data point to a critical role for regulatory rewiring through the serotype-specific mutation of regulator-encoding genes

(Lynskey *et al.*, 2013, Cao *et al.*, 2014, Lynskey *et al.*, 2015, Miller *et al.*, 2015a, Miller *et al.*, 2015b).

Strain-specific variation in GAS virulence has perhaps best been studied in relation to the selection of hyper-virulent derivatives during invasive infections (Engleberg *et al.*, 2001, Sumby *et al.*, 2006, Hollands *et al.*, 2010, Li *et al.*, 2014), the molecular basis of which is the mutation of regulator-encoding genes. The consequence of these mutations is an increased ability to evade the host immune response due to the enhanced expression, and/or the reduced degradation, of immunomodulatory virulence factors (Kwinn & Nizet, 2007). We will begin this review with a discussion of strain-specific regulatory gene mutations, and in particular mutations that lead to invasive disease hyper-virulence or enhanced carrier isolate status. We will subsequently discuss serotype-specific regulatory gene mutations, and in particular mutations that rewire regulation in serotype M3 isolates. In each case, we will review the function of the encoded proteins, the mutations that occur, and the transcriptional-, phenotypic-, and disease-specific consequences of these mutations. Given that regulatory genes have been identified as "hot-spots" for mutation in other bacteria (Yang *et al.*, 2011, Lieberman *et al.*, 2011), the information in this review may be applicable to variation observed for a broad swath of pathogens.

Strain-specific variation in regulatory activity

Mutation is a natural process that occurs at low frequency (calculated at ~10^{-9} mutations per generation in GAS) (Scott *et al.*, 2012) during replication of the bacterial chromosome. In general, mutations that are beneficial to the organism are maintained within the population whereas detrimental mutations are lost. However, this distinction is not always clear cut. For example, as we will discuss, during invasive GAS infections there is the selection for gene mutations that result in an increased ability to resist neutrophil-mediated killing. Given the beneficial nature of resisting killing by neutrophils it would be expected that many, if not all, GAS isolates harbored these mutations. This is not the case however, as the same mutations that result in an increased ability to resist neutrophil-mediated killing also result in a decreased ability of GAS to colonize and proliferate in the upper respiratory tract. As upper respiratory tract infections represent the majority of infections caused by GAS, this reduces the likelihood that GAS strains containing the regulatory gene mutations will be transmitted to other hosts. Thus, this in part explains why some mutations that, at least under some conditions, would seemingly be beneficial are only present in a strain-specific manner.

Strain-specific regulatory gene mutations that enhance GAS immune evasion

Following whole genome sequencing of 87 invasive disease serotype M3 GAS isolates, Beres *et al.* identified 22 genes with increased nucleotide diversity relative to the genome as a whole (Beres *et al.*, 2010). That some of these genes had higher levels of single nucleotide polymorphisms (SNPs) resulting in non-synonymous substitutions, rather than synonymous substitutions, is consistent with them undergoing diversifying selection (e.g. all 12 SNPs within the regulator of protease B [*ropB*, also known as *rgg*] gene result in non-synonymous changes) (Beres *et al.*, 2010). The *ropB* gene, along with the control of virulence regulator and sensor (*covR/S*, also known as *csrR/S*) genes, which are also undergoing diversifying

selection (Engleberg *et al.*, 2001, Mayfield *et al.*, 2014, Li *et al.*, 2014), represented three of the five genes with the highest mutation rate. Why mutations in *covR, covS*, or *ropB* are positively selected for will be discussed in this section.

The CovR/S two-component regulatory system—CovR/S form a two-component regulatory system, with CovS being a membrane-spanning sensor kinase that activates the cytoplasmically located CovR response regulator via phosphorylation, enhancing its ability to dimerize and bind DNA (Figure 1) (Graham *et al.*, 2002, Gryllos *et al.*, 2003, Gusa *et al.*, 2006). The CovR/S system is an atypical two-component system as it primarily negatively regulates gene expression, with ~10% of GAS transcripts having reduced abundance due to CovR/S activity. Included within these repressed transcripts are almost two dozen that encode for key GAS virulence factors, a common characteristic of which is that they enhance the ability of GAS to evade the host immune response. For example, the capsule has anti-phagocytic properties (Wessels & Bronze, 1994) while SpyCEP reduces neutrophil migration and activation by degrading chemokine gradients (Zinkernagel *et al.*, 2008). Given the function of CovR/S-repressed virulence factors, it is not surprising that this regulatory system is down-regulated during invasive GAS infections, enhancing virulence factor expression (Figure 1). Recent data indicates that the CovR/S system is down-regulated, at least in part, following binding of the human antimicrobial peptide LL-37 to the extracellular domain of CovS (Gryllos *et al.*, 2008, Velarde *et al.*, 2014). The interaction between LL-37 and CovS reduces CovS activity, which leads to a reduction in phosphorylated CovR.

The importance of reducing CovR/S activity during invasive infections is perhaps best exemplified by the fact that *covR/S* mutant strains are commonly selected for, and can be recovered from, patients with invasive infections (Ikebe *et al.*, 2010). That *covR/S* mutants are derived from parental strains with wild-type *covR/S* genes is evident from a study in which mice that were infected via the subcutaneous injection of wild-type GAS developed, in a time-dependent manner, abscesses containing a mixture of parental and *covR/S* mutant derivatives (Sumby *et al.*, 2006). Relative to the parental strains, *covR/S* mutants show increased lethality in murine models of bacteremia and soft tissue infection, and increased resistance to neutrophil-mediated killing. However, it should be stressed that not all invasive disease GAS isolates harbor *covR* or *covS* mutations, rather invasive infections appear to be caused by a mixture of parental and mutant GAS strains. We hypothesize that the large number of secreted virulence factors produced by the *cov* mutants have a positive bystander effect on neighboring parental GAS cells, enhancing the probability of parental cells disseminating to other hosts (Trevino *et al.*, 2009).

Interestingly, mutations occur at a higher frequency in *covS* than *covR*, even after adjusting for gene size (Friaes *et al.*, 2015). This is believed to be a consequence of the fact that *covS* and *covR* null mutant strains are not identical. For example, *covS* mutants produce undetectable levels of the secreted protease SpeB, while *covR* mutants produce high levels, two-fold higher than parental strains (Trevino *et al.*, 2009). We hypothesize that this is due to non-phosphorylated CovR being the form that is capable of binding to the *ropB* promoter and inhibiting its transcription, and subsequently preventing SpeB production. Thus, in a *covS* mutant strain non-phosphorylated CovR is highly abundant shutting off SpeB

expression, while in a *covR* mutant SpeB expression is not repressed (Figure 1). This hypothesis awaits experimental validation.

The differential regulation of SpeB expression is significant as SpeB targets a wide range of substrates for degradation, from host substrates such as anti-microbial peptides and chemokines, to many of the virulence factors that are part of the CovR/S regulon (Figure 2) (Carroll & Musser, 2011, Nelson *et al.*, 2011). That disrupting CovS activity is more advantageous than disrupting CovR activity is also evident from the analysis of *covR* mutant strains. While some naturally occurring *covR* mutants harbor null mutations, completely abrogating CovR function, others maintain repressor activity but are unable to interact with CovS, resulting in a regulatory pattern equivalent to a *covS* mutant (Trevino *et al.*, 2009).

Studies investigating the frequency of *covR/S* mutations identified between 30 and 50% of invasive GAS isolates, and between 2 and 5% of non-invasive isolates, harbor mutations (Ikebe *et al.*, 2010, Shea *et al.*, 2011). To help explain this infection-specific difference in *covR/S* mutation frequency, competition assays were performed between *covS* mutant and parental strains during growth in human saliva, an *ex vivo* model of an upper respiratory tract infection. It was identified that *covS* mutant strains are significantly outcompeted by parental strains during human saliva growth (Trevino *et al.*, 2009). Thus, it was concluded that the CovR/S system is critical to the ability of GAS to transition between infection types, with CovR/S activity contributing to the ability of GAS to cause non-invasive infections, and a reduction in CovR/S activity contributing to the ability of GAS to cause invasive infections. This was further supported by comparisons between *covS* mutant and parental strains in murine skin adherence and upper respiratory tract models of infection, with the *covS* mutants being attenuated in their ability to colonize (Hollands *et al.*, 2010, Alam *et al.*, 2013). Thus, while *covR/S* mutants are positively selected for during invasive infections due to their greater resistance to neutrophil-mediated killing, these mutant strains are not maintained in the population at high frequency due to their attenuated ability to cause non-invasive infections. Consequently, *covR/S* mutant strains that are recovered from invasive infections are, in most cases, thought to have arisen as a result of *de novo* mutations. In summary, the strain-specific mutation of *covR/S* has clinically relevant consequences, with these mutant strains enhancing the severity of invasive infections cause by this pathogen. However, as will be covered below, *covR/S* mutation is only one mechanism by which GAS may enhance virulence factor expression.

The regulator of protease B (RopB)—As mentioned above, a contributing factor behind the enhanced ability of *covS* mutant strains to cause severe invasive infections is thought to be the reduction in the expression of the SpeB protease, and therefore an enhancement in the abundance of secreted and cell-wall anchored virulence factors. If this were the case, mutation of the *speB* gene, or of a positive regulator of SpeB expression, could feasibly be other mechanisms by which GAS could achieve a so-called “invasive phenotype”. Consistent with this is the finding that the most polymorphic GAS gene identified in several population-based studies is *ropB* (Beres *et al.*, 2010, Friaes *et al.*, 2015), given that RopB is the major positive regulator of SpeB expression (Lyon *et al.*, 1998). During the transition from exponential to stationary phase, RopB binds to the *speB* promoter and dramatically upregulates transcription (Neely *et al.*, 2003) (Figure 1). However, unlike

the situation regarding *covR/S* mutations, there is currently a controversy as to which conditions promote the selection of *ropB* mutants (Kansal *et al.*, 2000, Ikebe *et al.*, 2010, Olsen *et al.*, 2015). Given that the GAS reservoir is the human upper respiratory tract, and that SpeB expression contributes to upper respiratory tract infection (Shelburne *et al.*, 2005), we cannot envision a scenario whereby *ropB* mutants would be selected for during pharyngeal infections. However, the substrate specificity of SpeB, which includes as many host immune system proteins as it does GAS anti-immune system virulence factors (Figure 2), does not provide an obvious case for the loss of SpeB expression being advantageous during invasive infections. We favor the view that GAS encounters multiple micro-environments during the course of an invasive infection, and that while SpeB may promote GAS virulence in some of these environments, there is at least one in which SpeB expression is strongly selected against. Further research is required to identify where and when *ropB* mutants arise.

Strain-specific regulatory mutations enhance the carrier isolate status of GAS

Many bacterial pathogens have the ability to asymptomatically colonize a host, although the molecular mechanisms that drive asymptomatic carriage, relative to symptomatic infection, are for the most part poorly defined. GAS is the prototype for studying asymptomatic carriage with, depending on the study, GAS carriage rates in the upper respiratory tracts of children ranging from 5 to 15% (Shaikh *et al.*, 2010). Animal models of infection comparing carrier and non-carrier GAS isolates identified that carrier isolates are attenuated in their ability to cause invasive infections (Krause *et al.*, 1962). Thus, there appears to be a genetic factor that distinguishes carrier GAS isolates from non-carrier isolates. Recent genome sequence comparisons of carrier and non-carrier GAS isolates have identified two regulatory networks whose disruption appear to contribute to the development of carrier isolates:

The multigene regulator in GAS (Mga)—Mga is the founding member of the Mga-like family of proteins that are present within multiple Gram-positive pathogens (e.g. the AtxA anthrax toxin regulator of *Bacillus anthracis*) (Hondorp & McIver, 2007, Hammerstrom *et al.*, 2015). That Mga contributes to GAS virulence was discovered more than 30 years ago (Spanier *et al.*, 1984). Mga positively regulates the expression of several classical GAS virulence factors, including the M protein and the C5a peptidase. In addition to regulating virulence factor expression, Mga regulates, and is also regulated by, carbohydrate metabolism (Ribardo & McIver, 2006). The regulation of Mga activity by carbohydrate metabolism occurs through the presence of two phosphotransferase system (PTS) regulatory domains (PRDs) located within Mga (Figure 3A). PTSs regulate carbohydrate transport and metabolism, and in part, this is achieved by the phosphorylation of PRD-containing proteins (Deutscher *et al.*, 2014). The importance of the PRDs to Mga activity will be further discussed later in the review, when we cover a strain-specific *mga* mutation that results in an alteration within one of these domains.

While characterizing a collection of carrier GAS isolates, it was discovered that two epidemiologically unassociated isolates both harbored the same 12 bp deletion within the *mga* promoter region (Flores *et al.*, 2013). The deletion removed one of two adjacent 12 bp direct repeats (Figure 3B), and likely occurred as a consequence of slipped-strand mispairing

during DNA replication. Analysis of the consequences of this deletion determined that it significantly reduced the transcription of both *mga* and Mga-regulated genes (e.g. a >20-fold decrease in *scpA* mRNA abundance), and reduced GAS virulence in animal models of necrotizing fasciitis (Figure 3C) (Flores *et al.*, 2013). It has been hypothesized, but not yet proven, that the observed 12 bp deletion is selected for among carrier strains as the decreased levels of extracellular protein expression, and in particular of the M protein, lowers the immunogenicity of these isolates during oropharyngeal colonization.

The LiaFSR three-component regulatory system—Where studied (e.g. *Streptococcus agalactiae* and *Enterococcus faecalis*), the Lia regulatory system has been identified as controlling species-specific responses to cell wall-active antibiotics and antimicrobial peptides (Klinzing *et al.*, 2013, Reyes *et al.*, 2015). A recent GAS study discovered that a non-synonymous mutation in the *liaS* gene, a putative sensor kinase, was one of three SNPs that distinguished GAS isolates recovered from a single individual during acute pharyngitis and subsequent asymptomatic carriage (Flores *et al.*, 2015). Through allelic exchange it was determined that the $liaS^{R135G}$ mutation enhanced characteristics associated with carriage (e.g. murine nasopharyngeal colonization), at the expense of characteristics associated with invasive infections (e.g. virulence in a murine necrotizing fasciitis model). While a full understanding of why the $liaS^{R135G}$ mutation results in the phenotypes observed remains to be determined, this mutation alters the transcription of multiple GAS genes, including *mga*, which is down-regulated. Thus, the data support the notion that the disruption of regulatory systems is an important contributor to the transition of GAS from disease to carriage states (Flores *et al.*, 2015). This may be a widespread phenomenon as similar findings have also been observed following comparison of disease and carrier isolates of pathogens such as *S. aureus* and *Neisseria meningitidis* (Schoen *et al.*, 2008, Young *et al.*, 2012).

A strain-specific mutation in *mga* enhances invasive GAS infections

A whole-genome sequencing study of serotype M59 GAS isolates identified a higher than expected number of SNPs in *mga* (Sanson *et al.*, 2015b). The most commonly occurring SNP, which arose independently at least five times, resulted in a H201R amino acid substitution. The H201 amino acid resides within the first of two PRD domains present within Mga (Figure 3A). To determine whether the H201R substitution altered Mga regulatory activity, comparisons were performed between parental and H201R isogenic mutant strains. The mutant strain had significantly increased expression of *mga* and Mga-regulated genes, altered regulation that resulted in the mutant strain having enhanced virulence in non-human primate and murine models of invasive GAS infection (Figure 3C) (Sanson *et al.*, 2015b). Recently, it was identified that the phosphorylation of two histidine residues located within the first Mga PRD domain, H207 and H273, reduces Mga activity (Hondorp *et al.*, 2013, Sanson *et al.*, 2015a). H201 is located on the same α-helix as H207, and is also in close proximity to H273 (Sanson *et al.*, 2015a). Thus, the data are consistent with the hypothesis that the H201R substitution enhances Mga activity by preventing the inhibitory phosphorylation of H207 and/or H273.

Serotype-specific variation in regulatory activity

As mentioned, epidemiological analyses have identified associations between certain GAS serotypes and disease phenotypes (Colman *et al.*, 1993). This implies that isolates of these serotypes are distinct, in gene content or gene regulation, from those of other serotypes and that these differences promote the ability of isolates of these serotypes to preferentially cause individual diseases. If so, the study of these serotypes may shed light on the virulence factors and regulatory patterns required for GAS to cause these diseases, information that may be useful with regard to the development of novel therapeutic or preventive regimes. While serotypes show variation in the assortment of bacteriophage and pathogenicity islands integrated into their genomes (Green *et al.*, 2005, Zhu *et al.*, 2015), none are unique to a single serotype. Therefore, we favor the hypothesis that it is the alternate regulation of core chromosomally-encoded virulence factors that is at the crux of GAS-serotype disease-phenotype associations.

Rewiring of regulatory networks in serotype M3 GAS isolates

Serotype M3 GAS isolates are associated with causing particularly severe invasive infections with a high mortality rate (Beres *et al.*, 2006). Investigations to gain insight into why isolates of this serotype are hyper-virulent during invasive infections have discovered that M3 isolates, since at least the 1920's, harbor mutations within at least three regulatory genes, *rocA, fasC*, and *rivR* (Cao *et al.*, 2014, Lynskey *et al.*, 2015, Miller *et al.*, 2015a). The type and consequences of mutations in these genes will be discussed in this section, including how they contribute to the observed invasive disease hyper-virulence of M3 isolates.

The regulator of Cov (RocA) protein—The *rocA* gene was discovered from a transposon mutagenesis screen in which *rocA* disruption enhanced the abundance of *covR/S* mRNA (Biswas & Scott, 2003). Given that RocA shares sequence similarity to membrane-spanning sensor kinases, it is likely that this protein enhances *covR* mRNA levels indirectly. Indeed, while a full understanding of RocA activity is lacking, recent data are consistent with RocA being a pseudokinase that functions through the CovR/S system, and that it does this by indirectly enhancing the phosphorylation status of CovR (Miller *et al.*, 2015a) (Sumby, unpublished).

Analysis of more than 450 serotype M3 GAS isolates, which were recovered between the 1920s and 2010, identified that they all harbor a frameshift mutation within *rocA* (Lynskey *et al.*, 2015, Miller *et al.*, 2015a). This mutation, a 1 bp deletion from within a homopolymeric tract (Figure 4A), truncates the protein within the c-terminal ATPase domain (Figure 4B). RNAseq analysis comparing a parental M3 isolate with a derivative in which a functional *rocA* allele had been introduced identified 56 mRNA transcripts that were differentially regulated (Miller *et al.*, 2015a). Importantly, a third of the differentially regulated genes encode for virulence factors, including the hyaluronic acid capsule (120-fold higher mRNA levels in the parental strain) and the chemokine protease SpyCEP (90-fold higher mRNA levels in the parental strain). Thus, the natural *rocA* mutation of serotype M3 GAS isolates is the molecular basis behind a significant upregulation in virulence factor expression by isolates of this serotype (see pink-shaded section of Figure 5). Virulence

assays identified that a parental M3 isolate could survive the bactericidal properties of human blood, and cause mortality in a murine bacteremia model of infection, at higher levels than a *rocA* complemented derivative (Miller *et al.*, 2015a). Thus, the *rocA* mutation contributes to the invasive disease hyper-virulence of serotype M3 GAS.

The fibronectin/fibrinogen-binding / hemolytic activity / streptokinase regulatory (Fas) system—The Fas system is encoded by a four gene locus, *fasBCAX* (Figure 4C), with FasB and FasC having sequence similarity to membrane-spanning sensor kinases, FasA having sequence similarity to response regulators, and FasX being a small regulatory RNA (sRNA). While FasBCA have not been studied in detail, all three are required for significant production of FasX, which is the effector molecule of this system (Kreikemeyer *et al.*, 2001). FasX is hypothesized to mediate the transition of GAS from the colonization to the dissemination stages of infection. This hypothesis is based upon the findings that FasX positively regulates streptokinase expression, a virulence factor that promotes blood clot degradation and tissue barrier destruction, and negatively regulates expression of the collagen-binding pilus and the fibronectin-binding adhesins PrtF1 and PrtF2 (Ramirez-Pena *et al.*, 2010, Liu *et al.*, 2012, Danger *et al.*, 2015a, Danger *et al.*, 2015b). FasX activity occurs at the post-transcriptional level (Miller *et al.*, 2014).

Comparisons of FasX abundance between isolates of eight different GAS serotypes identified that M3 isolates have lower levels relative to other serotypes (Perez *et al.*, 2009). Examination of the M3 *fas* locus uncovered a frameshift mutation in the *fasC* gene, leading to the premature termination of the gene (Figure 4C) and consequently truncation of the FasC protein (Figure 4D). The mutation, a deletion of one of three adjacent 4 bp (TTTA) repeats, was subsequently identified in all of 125 serotype M3 isolates that were recovered in a temporally (1920s to 2010) and spatially (Europe, North America, Japan, and Russia) diverse manner. Through use of complementation assays it was determined that the *fasC* mutation was responsible for the low abundance of FasX observed for serotype M3 isolates (Cao *et al.*, 2014).

It was hypothesized that the reduction in FasX abundance in serotype M3 isolates alters the expression of streptokinase, pilus, PrtF1, and PrtF2, and therefore M3 GAS virulence (see green-shaded region of Figure 5). While it has yet to be determined whether the adhesins are differentially regulated due to the *fasC* mutation, it has been confirmed that complementing this mutation enhances streptokinase expression (Cao *et al.*, 2014). Given that M3 isolates are hyper-virulent during invasive infections it seems counterintuitive that these isolates would harbor a mutation that reduces the expression of this crucial virulence factor. Importantly, while complementing the M3 *fasC* mutation increases streptokinase levels relative to the parental isolate, the parental isolate actually has higher levels relative to isolates of other serotypes. This is due to the fact that streptokinase expression is increased due to the M3 *rocA* mutation (Miller *et al.*, 2015a), and that this more than makes up for the "decrease" in expression due to the *fasC* mutation. Thus, if the *fasC* mutation contributes to the invasive disease hyper-virulence of M3 isolates then it likely does so through the altered expression of virulence factors other than streptokinase.

The RofA-like protein IV regulator (RivR)—RivR negatively regulates the expression of two virulence factors in serotype M1 GAS strains, the capsule and the protein G-related alpha-2-macroglobulin-binding protein (GRAB) (Trevino *et al.*, 2013). The anti-phagocytic properties of the capsule have already been discussed (Wessels *et al.*, 1991), while GRAB binds the human protease inhibitor alpha-2-macroglobulin and regulates proteolysis at the GAS cell surface (Rasmussen *et al.*, 1999). Analysis of the *rivR* gene in 125 serotype M3 isolates identified that the oldest isolate, from the 1920s, harbored three non-synonymous SNPs within RivR. The remaining 124 isolates, which were isolated between 1937 and 2010, contained an additional alteration in *rivR*, a 1 bp deletion within a homopolymeric tract (going from seven T's to six; Figures 4E and 4F). Through use of complementation assays it was determined that one of the three non-synonymous SNPs, leading to a L242P change, completely disrupts RivR activity in M3 isolates (Cao *et al.*, 2014). Thus, M3 isolates since at least the 1920s harbor null mutations in *rivR*.

The phenotypic consequences of *rivR* mutation in serotype M3 isolates are unclear. On the one hand, *rivR* disruption leads to the enhanced expression of GRAB, as expected (see beige-shaded region of Figure 5). On the other hand, while RivR negatively regulates capsule expression in M1 isolates, no such activity was observed following *rivR* complementation in an M3 isolate (Cao *et al.*, 2014). Unfortunately, as the mechanism by which RivR regulates capsule expression in M1 isolates has not been delineated this prevents assessment of why capsule is not RivR-regulated in a complemented M3 isolate. What is known however, is that RivR has no regulatory activity in a *covR* mutant strain, suggesting that RivR functions through CovR. A current hypothesis is that RivR binds to the *has* promoter and that this enhances CovR binding, leading to repression of capsule expression. Consistent with this is the finding that the *has* promoter of M3 isolates has multiple genetic alterations relative to that of M1 isolates, possibility providing an explanation as to why RivR does not regulate capsule expression in M3 GAS.

Summary of regulator-encoding gene mutations in serotype M3 GAS—In summary, data are consistent with serotype M3 GAS isolates causing disease over the course of the last 90-plus years all harboring mutations in *rocA, rivR*, and *fasC* regulator-encoding genes. Given that only serotype M3 GAS isolates have mutations within all three of these genes, indeed M3 isolates are the only serotype that harbor null mutations in *fasC or rivR*, we propose that in combination the three regulatory gene mutations are the driving force behind the serotype-specific virulence factor expression profile observed for M3 isolates. Interestingly, serotype M18 GAS isolates are also uniformly *rocA* mutants (but do not harbor mutations in *rivR* or *fasC* at any detectable frequency) but are not associated with causing severe invasive infections (Lynskey *et al.*, 2013). Thus, while *rocA* mutation is thought to be necessary for invasive disease hyper-virulence, and therefore the association of serotype M3 isolates with particularly severe invasive infections, it is not thought to be sufficient.

Conclusions

For an increasing number of bacterial pathogens, it is becoming apparent that regulatory gene mutations play an integral role in the frequency and severity of infections caused by

these pathogens. From disruption of the repressor of toxins (*rot*) gene in *S. aureus* USA500 strains (Benson *et al.*, 2014), to disruption of the positive regulatory factor of listeriolysin (*prfA*) gene in select isolates of *Listeria monocytogenes* (Becavin *et al.*, 2014, Rupp *et al.*, 2015), to disruption of the regulator of *lasB* (*lasR*) gene in *Pseudomonas aeruginosa* isolates during infection of the cystic fibrosis lung (Smith *et al.*, 2006, Diaz Caballero *et al.*, 2015), these mutations show clinical relevance. Perhaps in no pathogen has the incidence and consequences of natural regulatory gene mutations been more extensively studied than in GAS. Full genome sequences of thousands of GAS isolates that span multiple serotypes, diseases, sites of isolation, geographic locations, and years of isolation have enabled in-depth analyses of strain and serotype-specific variation (Maruyama *et al.*, 2016). As described in this review, mutations in both positive (e.g. Mga) and negative (e.g. CovR) regulators of virulence factor expression are associated with phenotypic variation in GAS. These mutations can dramatically alter disease potential, from promoting invasive infections to promoting asymptomatic carriage. Further, it should also be noted that the mutation-induced differences in the assortment of cell-surface and secreted proteins has the potential to significantly impact the efficacy of the GAS vaccines that are currently being tested.

As we move toward a future where infecting pathogens are whole-genome sequenced within a few hours of the patient arriving at the hospital, it may be prudent to determine the allelic status of the genes described in this review given their impact on disease potential. Such information may inform the clinician with respect to the potential of the strain to disseminate and cause more severe infections, and ultimately may influence treatment options. Even if this information is never used in the clinic, it would be valuable to the study of pathogen evolution and emergence, and we look forward to the challenges of drawing a more direct line between regulatory gene mutations and GAS disease manifestations.

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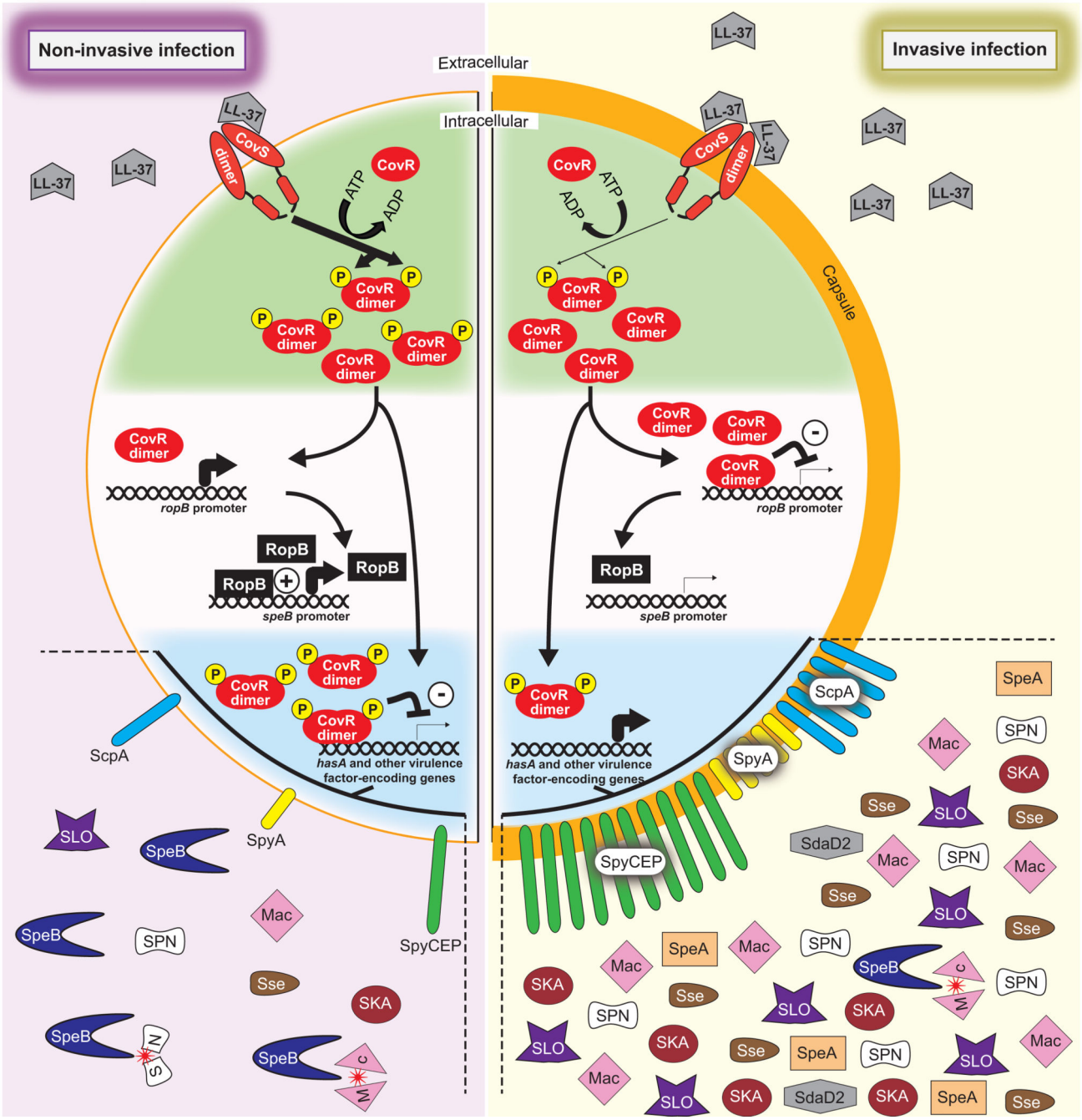

Figure 1. Differential regulatory activity of the CovR/S two-component system between invasive and non-invasive infections

Shown is a schematic of a GAS cell, with the left half of the cell showing CovR/S-mediated regulation that occurs during non-invasive infections, and the right half of the cell showing CovR/S-mediated regulation that occurs during invasive infections. The upper (green) section highlights the interactions between CovR and CovS. Binding of the human antimicrobial peptide LL-37 by CovS, which occurs at higher frequency during invasive infections, diminishes CovS kinase activity and hence CovR phosphorylation. The middle (white) section highlights the hypothesized regulation of SpeB protease expression, with

non-phosphorylated CovR being able to bind, only when at a sufficiently high concentration as is the case during invasive infections (or in a *covS* mutant), to the *ropB* promoter and repressing transcription. RopB is a transcription factor that is required for high level SpeB expression. The lower (blue) section highlights the consequences of CovR phosphorylation status with regard to the expression of multiple immunomodulatory virulence factors. The non-exhaustive list of CovR/S-repressed virulence factors shown are Mac-1-like protein (Mac), streptokinase (SKA), Streptolysin O (SLO), S. pyogenes NADase (SPN), secreted streptococcal esterase (Sse), Streptococcal pyogenic exotoxin A (SpeA), cysteine protease (SpeB), C5a peptidase (ScpA), chemokine protease (SpyCEP), S. pyogenes ADP-ribosylating toxin (SpyA), and group A streptococcal DNase D2 (SdaD2). The ability of SpeB to cleave and inactivate GAS virulence factors is also shown (red stars; see Figure 2 for more detail about SpeB activity).

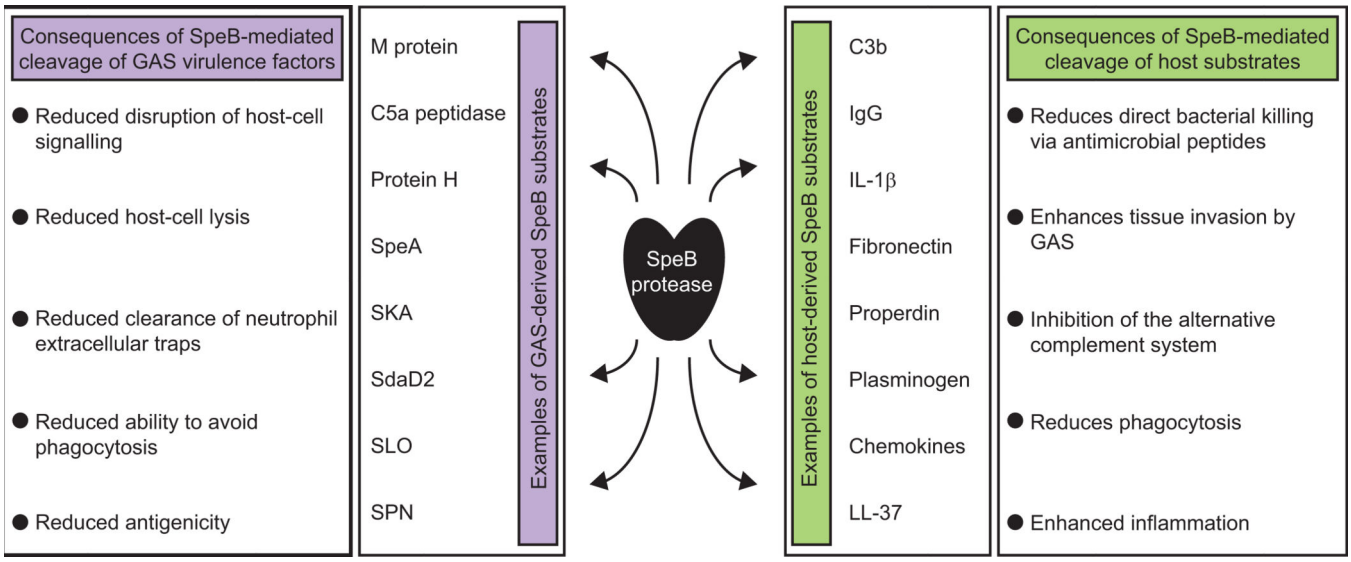

Figure 2. SpeB targets an array of host and GAS proteins for degradation

Shown are examples of host (green) and GAS (purple) proteins that are targeted for degradation by SpeB, as well as some of the consequences of this protease activity.

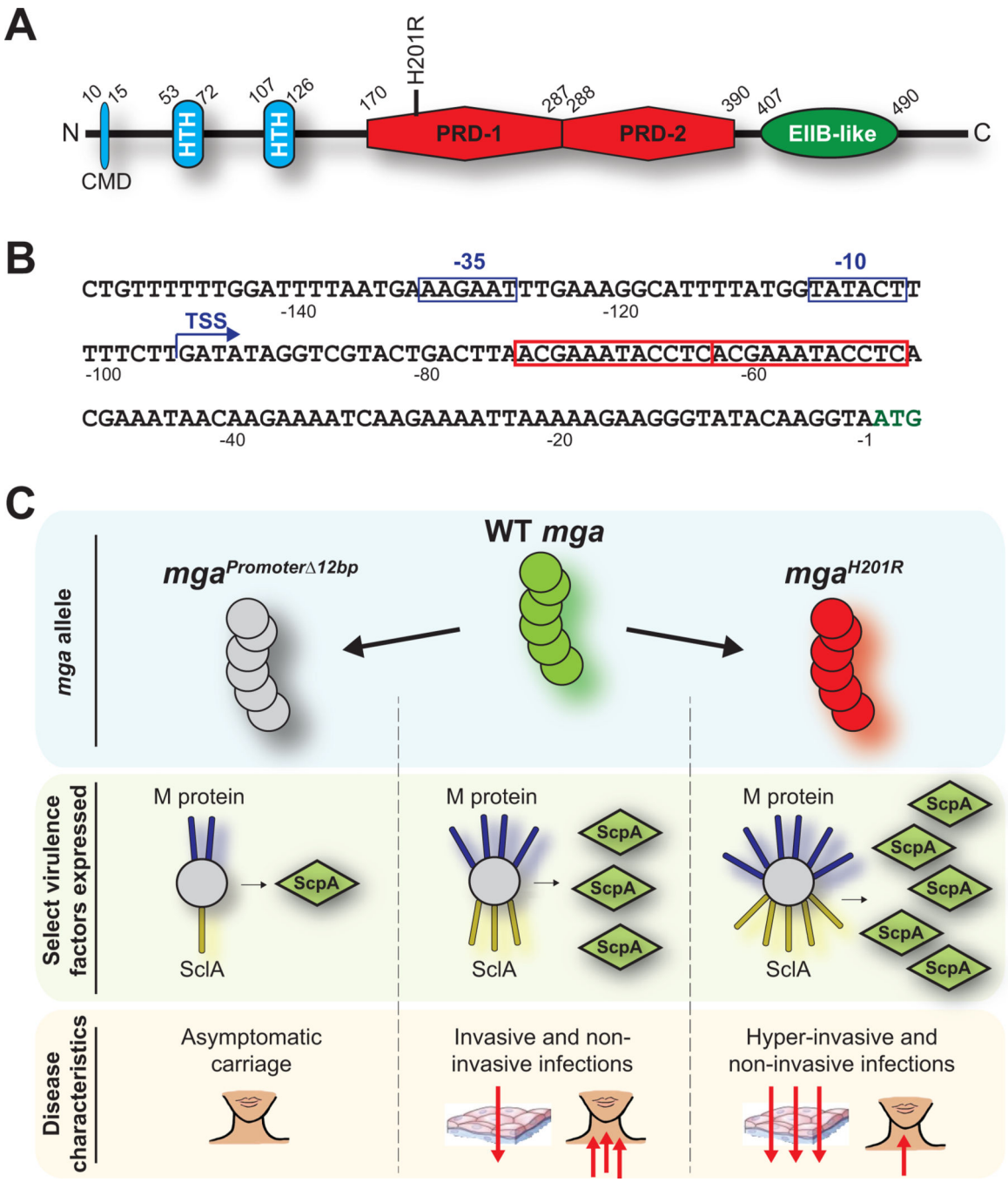

Figure 3. Distinct *mga* mutations lead to altered disease characteristics

(**A**) Domain structure of the Mga protein. The amino acids that boarder each domain are numbered. The identified domains are: conserved Mga domain (CMD), helix-turn-helix DNA-binding motif (HTH), phosphotransferase system regulatory domain (PRD), and a domain important in oligomerization (EIIB-like). The location of the H201R mutation is highlighted. (**B**) Nucleotide sequence of the *mga* promoter region. Nucleotides are numbered relative to the A of the ATG start codon (green nucleotides). The transcriptional start site (TSS) of the major *mga* promoter is shown with a bent arrow, and the −10 and −35 promoter

sequences are highlighted with blue boxes. In red boxes are the two 12 bp direct repeat sequences present upstream of *mga*, one of which is deleted in select carrier GAS isolates. (**C**) Regulatory and disease-specific consequences of select *mga* mutations. The number of red arrows highlight the severity of the infections.

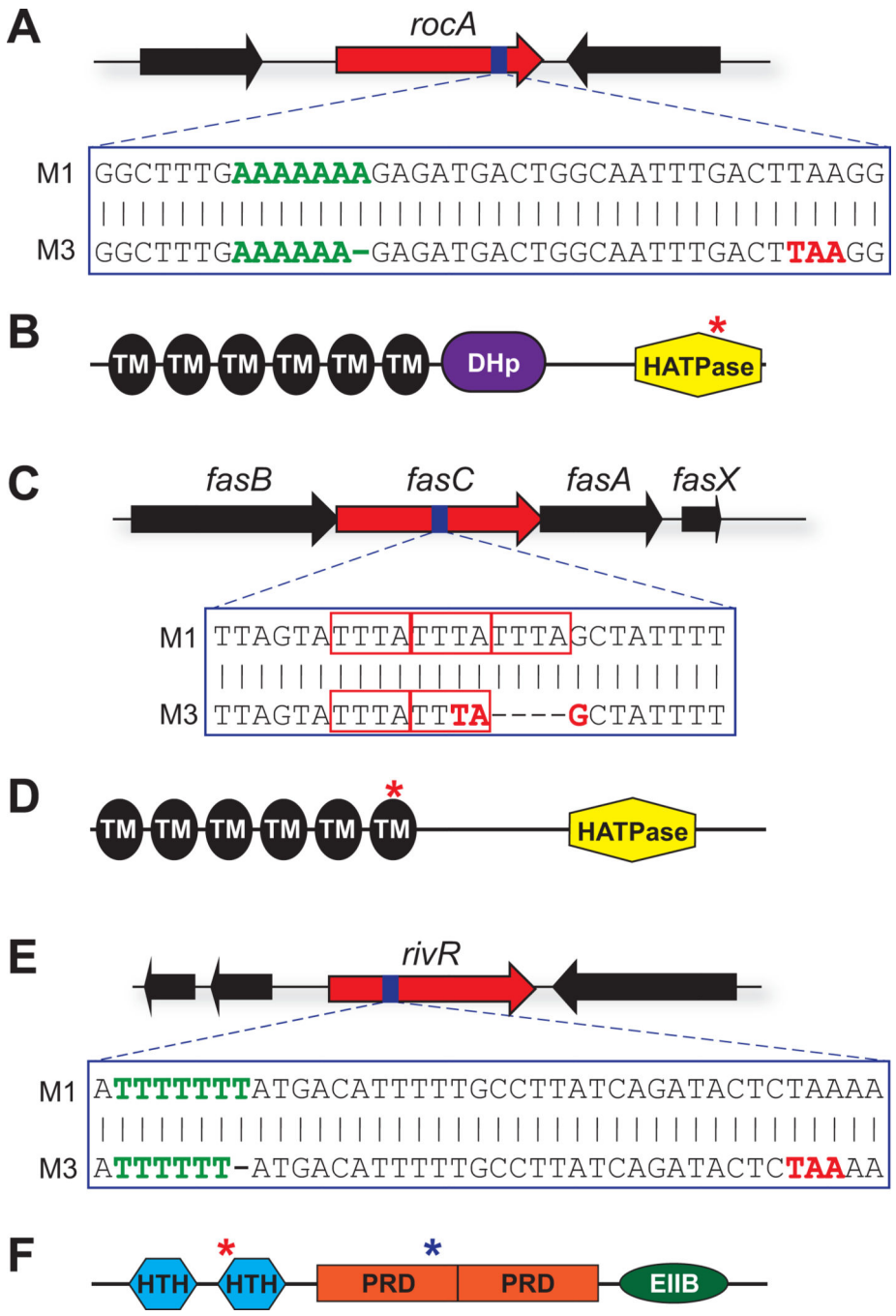

Figure 4. Serotype M3 GAS isolates harbor mutations in the *rocA*, *fasC*, and *rivR* regulator-encoding genes

(**A**) M3 isolates have a 1 bp deletion in *rocA*. Shown is a schematic of the *rocA* gene, with the region of the gene that contains the 1 bp deletion in serotype M3 isolates being highlighted with blue shading. The nucleotide sequence of this region is shown in a comparison between M1 and M3 isolates. The green nucleotides highlight the homopolymeric tract which contains the M3 GAS deletion, while the red nucleotides highlight the premature start codon which is introduced into the M3 GAS *rocA* gene due to

the deletion. (**B**) Domain analysis of the RocA protein. Identified domains are: transmembrane domains (TM; black), a dimerization and histidine phosphotransfer domain (DHp; purple), and a histidine-kinase-like catalytic domain (HATPase; yellow). The red asterisk highlights the location of the truncation that occurs in serotype M3 isolates. (**C**) M3 isolates have a 4 bp deletion in *fasC*. The red rectangles highlight the three tetra-nucleotide repeat sequences, one of which is deleted in serotype M3 GAS isolates. (**D**) Domain analysis of the FasC protein. The red asterisk highlights the location of the truncation that occurs in serotype M3 isolates. (**E**) M3 isolates have a 1 bp deletion in *rivR*. (**F**) Domain analysis of the RivR protein. RivR is a member of the Mga-like family of transcriptional regulators, and similar to Mga has helix-turn-helix DNA-binding motifs (HTH), phosphotransferase system regulatory domains (PRD), and a domain important in oligomerization (EIIB-like). The red asterisk highlights the location of the truncation that occurs in serotype M3 isolates. The blue asterisk highlights the location of the L242P amino acid substitution that occurs in M3 isolates.

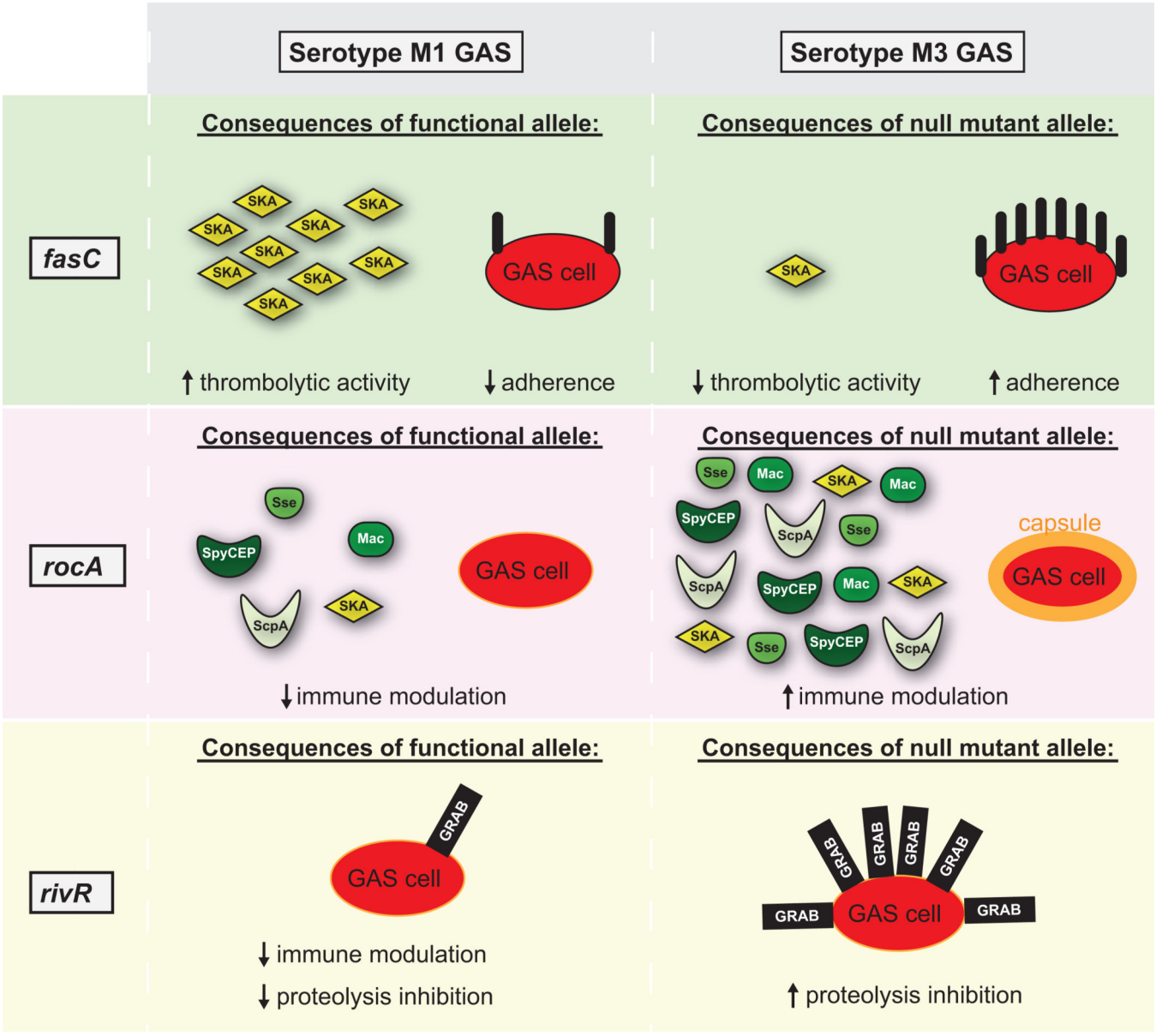

Figure 5. Serotype M3 GAS isolates have unique virulence characteristics as a consequence of the *fasC*, *rocA*, and *rivR* gene mutations

Shown is a comparison of virulence characteristics, and virulence factor expression, that occurs in serotype M1 and M3 GAS isolates due to the presence/absence of functioning FasC (green-shaded regions), RocA (pink-shaded regions) and RivR (beige-shaded regions) proteins. The Fas system: A functioning Fas system leads to the production of the sRNA FasX. FasX enhances the stability of mRNA encoding the thrombolytic agent streptokinase, and reduces the translation of mRNAs encoding adhesins. Due to the inactivation of the *fasC* gene, serotype M3 isolates do not have this regulatory activity. RocA: In serotype M1 isolates RocA enhances the activity of the CovR/S system which represses the expression of multiple immunomodulatory virulence factors. Due to inactivation of the *rocA* gene, serotype M3 isolates do not have this regulatory activity. RivR: In serotype M1 isolates RivR

represses transcription of genes encoding for the immunomodulatory capsule and cell-surface protease inhibitor GRAB. Due to inactivation of the *rivR* gene, serotype M3 isolates do not have this regulatory activity (although, for unknown reasons, a functional RivR has no regulatory effect on the capsule in the M3 background).