



# Identification and Characterization of Serotype-Specific Variation in Group A *Streptococcus* Pilus Expression

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**ABSTRACT** Isolates of a given bacterial pathogen often display phenotypic variation, and this can negatively impact public health, for example, by reducing the efficacy of preventative measures. Here, we identify that the human pathogen group A *Streptococcus* (GAS; *Streptococcus pyogenes*) expresses pili on its cell surface in a serotype-specific manner. Specifically, we show that serotype M3 GAS isolates, which are nonrandomly associated with causing particularly severe and lethal invasive infections, produce negligible amounts of pili relative to serotype M1 and M49 isolates. Performance of an interserotype transcriptome comparison (serotype M1 versus serotype M3) was instrumental in this discovery. We also identified that the transcriptional regulator *Nra* positively regulates pilus expression in M3 GAS isolates and that the low level of pilus expression of these isolates correlates with a low level of *nra* transcription. Finally, we discovered that the phenotypic consequences of low levels of pilus expression by M3 GAS isolates are a reduced ability to adhere to host cells and an increased ability to survive and proliferate in human blood. We propose that an enhanced ability to survive in human blood, in part due to reduced pilus expression, is a contributing factor in the association of serotype M3 isolates with highly invasive infections. In conclusion, our data show that GAS isolates express pili in a serotype-dependent manner and may inform vaccine development, given that pilus proteins are being discussed as possible GAS vaccine antigens.

**KEYWORDS** phenotypic variation, gene regulation, *Streptococcus pyogenes*, pili, bacterial virulence

Bacterial pathogens often show significant intraspecies phenotypic variation, with colony morphology, growth requirements, adherence, and disease manifestations being just a few of the variable phenotypes described (1, 2). In some instances, phenotypic variation can be divided along type-specific (e.g., serotype, clonal type, haplotype) lines, indicating that there are type-specific genetic components responsible for the observed phenotypic differences (3, 4). For example, *Staphylococcus aureus* isolates from pulsed-field type USA300 show enhanced virulence relative to isolates from pulsed-field type USA200, and this is in part explained by differences in the Agr regulatory system, which is a major regulator of virulence factor expression (5, 6). In another example, the group A *Streptococcus* (GAS; *Streptococcus pyogenes*) shows serotype-specific variation in virulence and disease manifestations (7–9).

GAS isolates cause more than 700 million human infections globally per year, the majority of which are self-limiting infections of the upper respiratory tract (10). However, GAS isolates also cause severe invasive infections, including streptococcal toxic shock syndrome and necrotizing fasciitis (11). GAS infections may also result in one of

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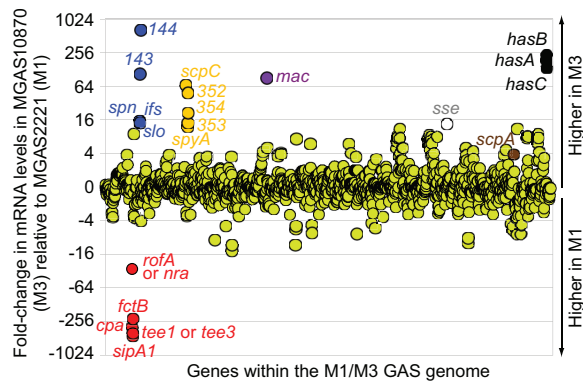
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several debilitating postinfection sequelae, such as acute rheumatic fever (12) or acute poststreptococcal glomerulonephritis (13). GAS isolates are grouped into serotypes on the basis of the sequence of the 5' end of the *emm* gene, a gene which encodes the classical virulence factor, the M protein (14). While all GAS isolates are thought to have the potential to cause all GAS diseases, there are several long-known GAS serotype-disease phenotype associations. For instance, serotype M3 isolates are nonrandomly associated with causing particularly severe and lethal invasive infections (15), while serotype M18 GAS isolates are nonrandomly associated with cases of acute rheumatic fever (16).

In the process of investigating the association of serotype M3 GAS isolates with severe invasive infections, we and others identified that since at least the 1920s M3 isolates have harbored null mutations in three regulator-encoding genes (*rocA*, *rivR*, and *fasC*) (7, 17, 18). These mutations result in a significant reassortment of virulence factor expression in M3 isolates, including expression of the antiphagocytic hyaluronic acid capsule (7), the human protease inhibitor-binding protein G-related  $\alpha_2$ -macroglobulin-binding protein (GRAB) (17), and the thrombolytic factor streptokinase (19). For at least one regulatory gene mutation (*rocA*), the altered regulation enhances the ability of M3 GAS to survive in human blood and cause disease in mouse models of invasive infection (7). The data are consistent with the regulatory consequences of these mutations, with a serotype M3 GAS-specific virulence factor profile being the driving force behind the association of M3 GAS with severe invasive infections.

Pili are cell surface proteins that have long been a subject of study due to their role in promoting bacterial adherence (20, 21). In what was a surprise to the field, it was reported in 2005 that GAS isolates express pili (22). Pili facilitate GAS binding to host cells/tissues, promote biofilm formation, and modify the capacity of GAS to evade the host immune system in what appears to be a serotype-specific fashion (22–26). Thus, pili play key roles in the ability of GAS to cause disease. The pilus biosynthesis genes are found within a region of the genome that is both polymorphic and polygenic, a region referred to as the fibronectin-binding, collagen-binding, T-antigen (FCT) region (27). There are nine known FCT types, and in almost all cases, isolates of any given serotype harbor the same FCT region (28). The pilus biosynthesis genes are under the regulatory control of a transcription factor encoded by a sequence within the FCT region. Depending on the FCT type, this regulatory protein is one of two orthologues, the regulator of F (RofA) or negative regulator of GAS (Nra), which share 62% amino acid sequence identity (29). How Nra regulates pilus expression differs in a strain- and/or serotype-specific manner. Podbielski et al., working in a serotype M49 background (FCT type 3 [FCT-3]), described Nra to be a negative regulator of pilus expression (30), while Luo et al., working in a serotype M53 background (FCT-3), identified Nra to be a positive regulator of pilus expression (31). FCT types 2, 3, and 4 also encode a second transcription factor, the multiple sugar metabolism regulator (MsmR) (32). Similar to RofA/Nra, there are strain- and/or serotype-specific differences as to whether MsmR positively or negatively regulates pilus expression (31, 32). Importantly, while published data suggest that there may be serotype-specific differences in how pili are expressed (29–31) and in how pili function (25, 33, 34), no data point to there being serotype-specific differences on whether or not pili are expressed.

Here, we identify that pili are expressed on the GAS cell surface in a serotype-specific manner. Specifically, relative to serotype M1 and M49 GAS isolates, M3 isolates produce negligible amounts of pili. Investigation into the mechanism behind the diminished pilus expression of M3 isolates identified that Nra is a positive regulator of the pilus in isolates of this serotype and that attenuated *nra* transcription underlies the pilus phenotype. We also demonstrate that MsmR has no effect on pilus expression in M3 GAS isolates. Low levels of pilus expression were shown to reduce the adherence of M3 isolates in a tissue culture-based model of adherence and to increase the ability of M3 strains to survive and replicate in nonimmune whole human blood. Given these phenotypes, we propose that the serotype-specific reduction in pilus expression is a



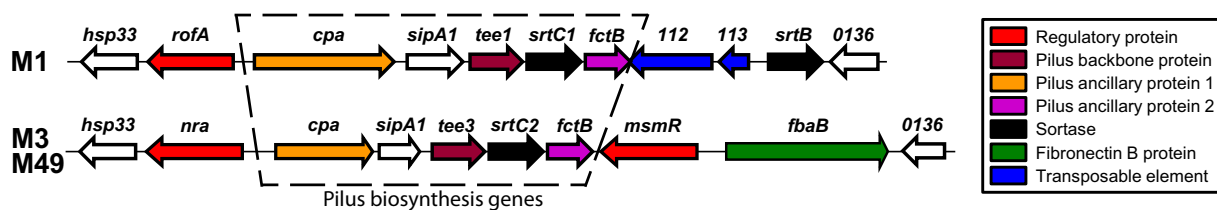
**FIG 1** Interserotype transcriptome comparison. Scatterplot graph depicting differential gene expression between representative serotype M1 (MGAS2221) and M3 (MGAS10870) isolates. Triplicate cultures of each strain were grown to the mid-exponential phase of growth in THY broth. Total RNA was isolated and subjected to RNA-Seq analysis. The relative expression levels of all genes present in both M1 and M3 GAS strains are graphed, with each one being represented by a circle. Select virulence factor-encoding genes are labeled, and the color of the circle is changed from green. Genes encoding pilus regulatory and biosynthesis proteins are colored red. Genes are arranged in the same order in which they appear in the chromosome.

factor contributing to the association of M3 GAS isolates with severely invasive and lethal infections.

## RESULTS

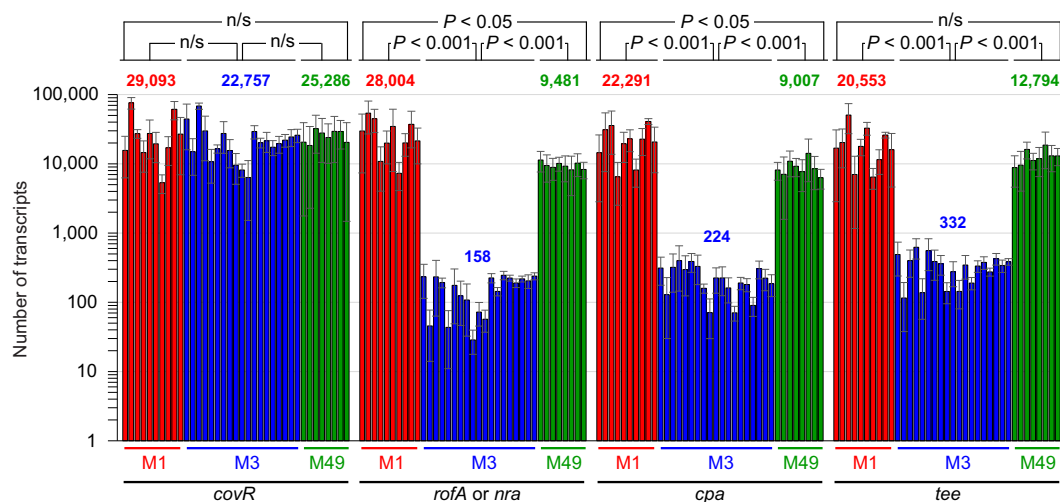
**Significant interserotype variation in the abundances of virulence factor-encoding mRNAs.** Epidemiological studies have identified that serotype M3 GAS isolates are nonrandomly associated with particularly severe and lethal invasive infections (35). We propose that an M3 GAS-specific transcriptional pattern, due partly to inactivating mutations in the *rocA*, *rivR*, and *fasC* regulator-encoding genes (7, 17), is the major contributor to the differential disease profile of M3 isolates. To further investigate possible serotype M3 GAS-specific variations in gene transcription, but this time investigating it at the transcriptome level, we compared the transcriptomes of representative serotype M1 and M3 GAS isolates via a transcriptome sequencing (RNA-Seq)-based approach. GAS isolates MGAS2221 (serotype M1) and MGAS10870 (serotype M3), which are representative of their respective serotypes, as evident from several large population-based studies (36, 37), were compared. A total of 210 mRNAs were differentially regulated between MGAS2221 and MGAS10870 at a 2-fold cutoff level (Fig. 1; see also Table S1 in the supplemental material). Importantly, a majority of the genes with the highest disparity in mRNA levels between the two isolates encode known or hypothesized virulence factors. Given that M1 GAS strains are *rocA* positive and M3 GAS strains are *rocA* negative, the finding that the capsule biosynthesis operon (*hasABC*; black circles in Fig. 1) was transcribed at a higher level in M3 isolates than M1 isolates was expected since RocA negatively regulates this operon (7, 18). However, an unanticipated finding was that the levels of mRNAs from the pilus biosynthesis locus (red circles in Fig. 1) were dramatically (230- to 463-fold) lower in the M3 isolate. Similarly, the level of expression of the mRNA encoding the regulatory protein Nra was 29-fold lower in the M3 isolate than the level of expression of *rofA* mRNA in the M1 isolate. Together, these data raise the possibility that a distinguishing attribute of serotype M3 GAS isolates is that they do not harbor pili on their cell surface at appreciable levels.

**Serotype M3 GAS isolates have a reduced level of transcripts from the pilus biosynthesis region.** We next assessed whether the low level of pilus gene expression in MGAS10870, as identified from the RNA-Seq analysis (Fig. 1), is a serotype-defining characteristic of M3 isolates. To achieve this we performed absolute quantification of select pilus (test) and nonpilus (control) transcript levels by quantitative reverse transcription-PCR (qRT-PCR). We compared the expression levels among 10 clinical M1 GAS isolates (recovered between 1973 and 2010 from Finland, Canada, Denmark, the



**FIG 2** FCT region of serotype M1, M3, and M49 isolates. The schematic shows the GAS pilus biosynthesis locus in isolates of the M1 and M3/M49 serotypes. Genes are represented by arrows, with the direction of the arrows mirroring the direction of transcription. The colors of the arrows highlight the functions of the encoded proteins (see the key).

United States, Australia, or the Czech Republic), 19 clinical M3 GAS isolates (recovered between 1937 and 2010 from Germany, the United, Canada, the United Kingdom, or undefined locations), and 8 clinical M49 GAS isolates (recovered between the 1970s and 2004 from Germany, the Czech Republic, the United States, or undefined locations) (see Table S2 for more information about the GAS strains used). We included serotype M49 isolates in the analysis due to the fact that they share the same FCT type as M3 isolates (FCT-3) (Fig. 2), but unlike for M3 isolates, the presence of pili on the surface of M49 GAS isolates has been well characterized (38). Note that serotype M1 isolates have an FCT-2 type and, hence, differ from M3 and M49 isolates by harboring the regulatory gene *rofA* and not *nra* and lacking the second regulatory gene, *msmR* (Fig. 2). As expected, given that the regulator-encoding gene *covR* is not part of the FCT region, no differences in the abundance of *covR* mRNA were observed among the M1, M3, and M49 isolates (Fig. 3). In contrast, statistically significant differences in the abundance of mRNAs from genes encoding the pilus regulatory gene (*rofA* or *nra*), a minor pilus protein (*cpa*), or the major pilus protein (*tee*) were observed between the M3 isolates and the serotype M1 and M49 isolates (Fig. 3). The pilus transcripts were present in the M3 isolates at levels between 39- and 177-fold lower than the levels observed in the M1 or M49



**FIG 3** Pilus biosynthesis and regulatory genes are transcribed at a significantly lower level in serotype M3 isolates than in serotype M1 or M49 isolates. TaqMan-based absolute quantification was performed to enable comparison of the abundance of mRNAs encoding the major (*tee*) and minor (*cpa*) pilus proteins and pilus regulatory proteins (*rofA* or *nra*) and the abundance of the regulator-encoding gene *covR* (as a control) in 10 clinical M1, 19 clinical M3, and 8 clinical M49 GAS isolates. The abundances of the mRNAs were determined from a minimum of duplicate exponential-phase GAS cultures of each strain, and the experiment was performed at least in duplicate. Each bar represents the mean  $\pm$  standard deviation for each strain, with the serotype M1 isolates indicated in red (in the following order from left to right, respectively: MGAS2221, MGAS313, MGAS1264, MGAS1508, MGAS22259, SF370, MGAS5005, MGAS3350, MGAS5456, and MGAS9127), the M3 isolates indicated in blue (in the following order from left to right, respectively: MGAS315, MGAS10870, MGAS491, MGAS159, MGAS15049, MGAS182, MGAS1254, MGAS1428, MGAS9056, MGAS9507, MGAS22283, 31616/3065, 90-254, 90-132, L-035, PD-824, Di944, Di1216, and Di1253) and the M49 isolates indicated in green (in the following order from left to right, respectively: 49/123, 591, 71-716, 90-053, 90-397, 88-299, 89-288, and 90-306). The mean for the grouped M1, M3, and M49 samples is also shown for each transcript investigated. Statistical significance was determined via the *t* test. n/s, not significant.

isolates. Thus, at least relative to serotype M1 and M49 GAS isolates, serotype M3 isolates transcribe the genes required for pilus biosynthesis at significantly reduced levels.

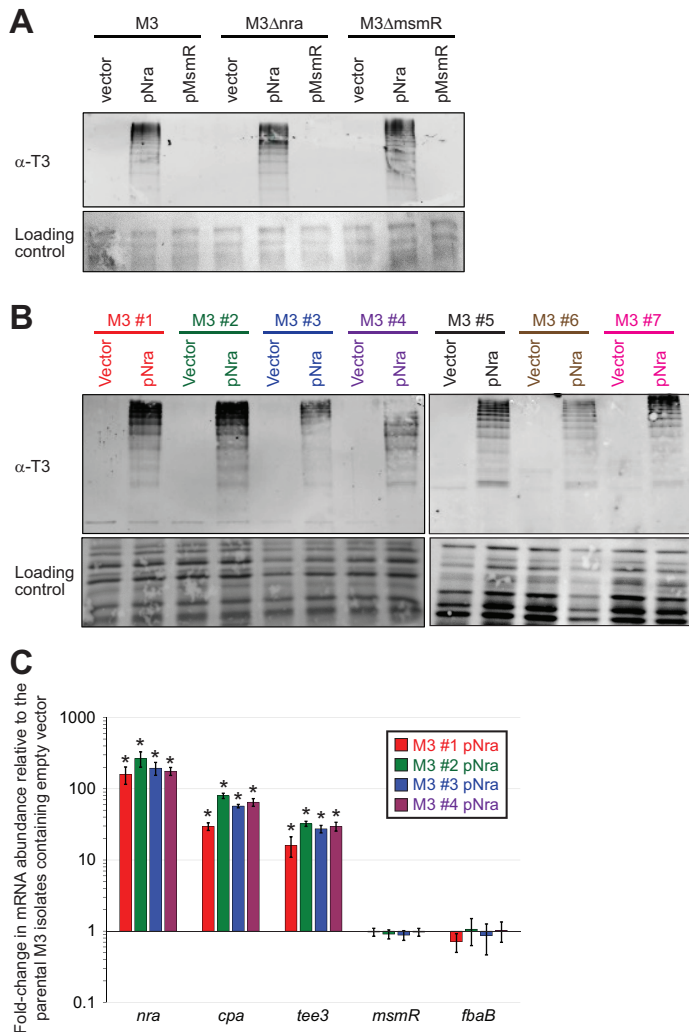
**Pili are not detected on the surface of clinical M3 GAS isolates but can be detected following the addition of an *nra*-expressing plasmid.** Given the published data showing the strain and/or serotype-specific variation in the roles that *Nra* and *MsmR* have in controlling pilus expression (30–32), we investigated this in M3 GAS isolates by creating *nra* and *msmR* deletion mutant derivatives of our parental M3 isolate MGAS10870. We also created plasmids pNra and pMsmR, which harbor, respectively, *nra* and *msmR* alleles similar to those observed in serotype M49 GAS isolates. We chose to work with M49 GAS alleles, as isolates of the M49 serotype are known to express pili (38) and these genes lack any serotype-specific mutations (see later). The pNra and pMsmR plasmids and the empty vector were individually transformed into the MGAS10870, MGAS10870  $\Delta nra$ , and MGAS10870  $\Delta ms mR$  backgrounds. These strains were grown to the exponential phase of growth, cell wall-anchored proteins were isolated, and the proteins were used in Western blot analysis with an antipilus (T3) antibody. No pilus-specific reactivity between the antibody and the protein samples from any strain that harbored either the vector or pMsmR was observed (Fig. 4A). In contrast, all three derivatives containing pNra expressed high levels of pili on their surface. The data are consistent with clinical M3 GAS isolates expressing negligible amounts of cell surface pili, with *Nra* positively regulating M3 GAS pilus expression and with *MsmR* having no effect on pilus expression in this serotype.

To ensure that our data gained with MGAS10870 were representative, we introduced the empty vector and pNra into six additional M3 isolates and tested the isolates for pilus expression via Western blot analysis. Similar to the data for MGAS10870, none of the additional M3 isolates containing the empty vector produced detectable pili, while the pNra-containing derivatives had robust pilus expression (Fig. 4B). Thus, the absence of detectable pilus expression by clinical M3 isolates and the dramatic increase in pilus expression in these isolates following the addition of plasmid pNra appear to be characteristic of serotype M3 GAS.

***Nra* positively regulates M3 GAS pilus expression at the transcriptional level.**

To assess whether the regulation of pilus expression by *Nra* in M3 GAS occurs at the transcriptional or posttranscriptional level, we used quantitative RT-PCR analysis. Comparing four strain pairs harboring the empty vector or pNra, we identified that all pNra-containing derivatives showed enhanced abundances of mRNAs encoding the major pilus protein (*tee3*; mean increase, 26-fold) and a minor pilus protein (*cpa*; mean increase, 58-fold) (Fig. 4C). The abundances of *msmR* and *fbaB* mRNAs were unaffected, while *nra* mRNAs were present at levels an average of 199-fold higher than those in the empty-vector-containing strains, a finding which equates to a level of *nra* mRNA abundance in clinical M3 isolates ~3-fold higher than that observed in clinical M49 isolates. In combination with the data in Fig. 3, our data are consistent with the lack of pilus expression by clinical serotype M3 isolates being a consequence of a low level of expression of *Nra*, a protein that positively regulates transcription of the pilus biosynthesis genes.

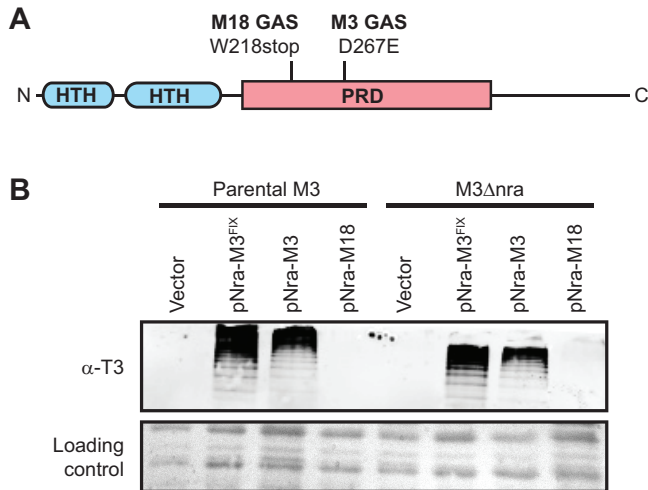
**M3 and M18 GAS isolates harbor mutations within *nra*.** We next turned our attention to investigating why serotype M3 isolates transcribe *nra* at a level significantly lower than the levels at which M1 GAS isolates transcribe *rofA* and M49 GAS isolates transcribe *nra*. One possibility could be that *Nra* positively regulates its own expression and that the M3 *nra* allele produces an afunctional protein. To look at this, we compared the *nra* alleles from isolates of different GAS serotypes and identified that the M3 allele contains a serotype-specific single nucleotide polymorphism (SNP). The M3-specific *nra* SNP, identified in the sequenced M3 isolates MGAS315 and SSI-1 (39, 40), results in a nonsynonymous amino acid change (D267E) compared to the sequences of other *nra* alleles (Fig. 5A). We sequenced *nra* in seven additional M3 strains (Table S2), isolated in a temporally and geographically disparate fashion, and determined that the M3-specific *nra* mutation was present in them all. In the process of



**FIG 4** Plasmid-expressed *nra*, but not plasmid-expressed *msmR*, enhances pilus expression in serotype M3 GAS isolates. (A) Western blot analysis. The parental M3 isolate MGAS10870 (M3), an *nra* deletion mutant derivative (M3Δ*nra*), and an *msmR* deletion mutant derivative (M3Δ*msmR*) were transformed with the empty vector or plasmids expressing Nra (pNra) or MsmR (pMsmR). Cell wall proteins from exponential-phase cultures were isolated and used with an anti-T3 antibody. Note the characteristic laddering pattern. The membrane was stained following protein transfer to serve as a loading control. (B) Western blot analysis. Seven serotype M3 GAS isolates were transformed with the empty vector or pNra, and cell wall proteins were analyzed by Western blotting. The isolates were MGAS22283 (M3 #1), MGAS9056 (M3 #2), MGAS15049 (M3 #3), MGAS10870 (M3 #4), MGAS491 (M3 #5), MGAS159 (M3 #6), and MGAS182 (M3 #7). (C) TaqMan-based quantitative RT-PCR. Derivatives of MGAS22283 (M3 #1), MGAS9056 (M3 #2), MGAS15049 (M3 #3), and MGAS10870 (M3 #4) containing either the empty vector or pNra were grown to the exponential phase of growth before they were harvested and analyzed via quantitative RT-PCR. Shown are the averages ± standard deviations for duplicate samples run in duplicate. \*, statistically significant difference relative to the parental isolates containing empty vector (*t* test, *P* < 0.05).

comparing *nra* alleles across serotypes, we also identified that the serotype M18 strain MGAS8232 harbors a premature stop codon in *nra* (Fig. 5A). Whether this mutation is encoded by the genomes of all serotype M18 GAS isolates and the regulatory consequences of this for pilus expression in M18 GAS are under investigation.

**The M3 and M49 *nra* alleles, but not the M18 *nra* allele, enhance pilus expression in M3 GAS when overexpressed.** To test whether the M3 and M18 *nra* alleles produce functional proteins, we created plasmids pNra-M3 and pNra-M18, respectively. We also constructed a derivative of pNra-M3 in which the D267E mutation was fixed, plasmid pNra-M3<sup>FIX</sup>, to enable us to assay the effects of this polymorphism on Nra activity. All three plasmids were transformed into MGAS10870 and its derivative,



**FIG 5** The M3 *nra* allele promotes pilus expression when expressed from a low-copy-number plasmid. (A) The 511-amino-acid Nra protein shares a domain architecture similar to that of other Mga-family members, with helix-turn-helix (HTH; blue) DNA-binding domains at their N termini, one or more centrally located phosphotransferase system regulatory domains (PRD; pink), and unstructured C termini. Highlighted are the locations of the M3 and M18 serotype-specific changes in the Nra protein, which are an aspartate-to-glutamate change or a truncation, respectively. (B) Western blot analysis. The parental M3 isolate (MGAS10870) and *nra* deletion mutant derivative (M3 $\Delta$ nra) were transformed with the empty vector or plasmids harboring the M3 *nra* allele (pNra-M3), a modified M3 *nra* allele in which the M3-specific SNP has been fixed (pNra-M3<sup>FIX</sup>), or the M18 *nra* allele (pNra-M18). Cell wall proteins were isolated from exponential-phase cultures and visualized by Western blotting using an anti-T3 antibody. The membrane was stained following protein transfer to serve as a loading control.

M3 $\Delta$ nra, and the six strains, along with vector-containing derivatives, were evaluated for pilus expression by Western blotting. Similar to the vector-containing strains, the strains carrying pNra-M18 failed to express detectable levels of pili (Fig. 5B), consistent with the presence of a premature stop codon in the MGAS8232 *nra* gene, resulting in an inactive Nra protein. However, all strains containing either pNra-M3 or pNra-M3<sup>FIX</sup> expressed cell surface pili (Fig. 5B). As plasmid pNra-M3 induces pilus expression, the D267E mutation within the M3 GAS *nra* allele is not a null mutation. While it is not a null mutation, we observed a small but reproducible increase in pilus expression by strains containing pNra-M3<sup>FIX</sup> relative to those containing pNra-M3. This is consistent with the D267E mutation marginally reducing the activity of the Nra protein. Given that the difference in activity was only minor, this mutation cannot be the main factor behind the low level of *nra* transcription in M3 isolates. Further evidence that the D267E mutation somewhat reduces Nra activity but is not the critical reason why M3 isolates produce very low levels of pili was gained by chromosomally fixing the D267E mutation within the MGAS10870 *nra* gene. The resultant strain, M3nra<sup>FIXED</sup>, expressed very low but detectable levels of cell surface pili, in contrast to the parental strain (Fig. S1).

**The low abundance of *nra* mRNA in serotype M3 GAS is not a consequence of an upstream VNTR.** Variable-number tandem repeats (VNTRs) are nucleotide repeat sequences that can increase or decrease in number, and when they are located within a promoter region, the number of repeats can influence gene transcription and/or mRNA translation. Indeed, it was recently shown that a VNTR upstream of *rocA* in GAS controls the translation of mRNA from this regulator-encoding gene (41). We identified a polynucleotide tract within the *nra-cpa* intergenic region that differed, in a serotype-specific manner, in the number of nucleotides that were present. In M3 GAS isolates there are eight A nucleotides, while in M49 GAS isolates there are seven (Fig. S2A). To assess whether this one-nucleotide difference could be behind the reduced levels of pilus expression in M3 GAS isolates, we created an MGAS10870 derivative in which we deleted an A nucleotide, to bring the number into line with that observed in M49 GAS isolates. Comparison of this derivative with the parental strain identified no difference

in pilus expression (Fig. S2B), and hence, the VNTR does not seem to influence pilus expression.

**CovR is a contributing factor in the serotype-specific regulation of pilus expression.** Previously, it was reported that mutation of *covR*, which encodes the response regulator component of the CovR/S two-component regulatory system, enhances pilus expression (42, 43). We investigated this in our representative serotype M1 (MGAS2221) and serotype M3 (MGAS10870) backgrounds. Through TaqMan-based qRT-PCR analysis, we identified that the *covR* mutation increased the abundance of *nra*, *cpa*, and *tee3* transcripts in our M3 background (Fig. 6A) but did not alter the abundance of *rofA*, *cpa*, or *tee1* transcripts in our M1 background (Fig. 6B). Western blot analysis of cell wall proteins determined that low but detectable levels of pili were observed in the strain M3Δ*covR* background (Fig. 6C). Additionally, we determined that disruption of *covS* (strain M3Δ*covS*) or introduction of a functional *rocA* gene (strain M3rocA<sup>FIXED</sup>) did not alter pilus expression, which, given that CovS and RocA enhance the abundance of phosphorylated CovR (7, 44), is consistent with nonphosphorylated CovR binding and repressing M3 GAS pilus expression. Our data show that CovR represses pilus expression in a serotype-specific manner and that while this contributes to the low level of pilus transcription in M3 isolates, it does not appear to be the major factor.

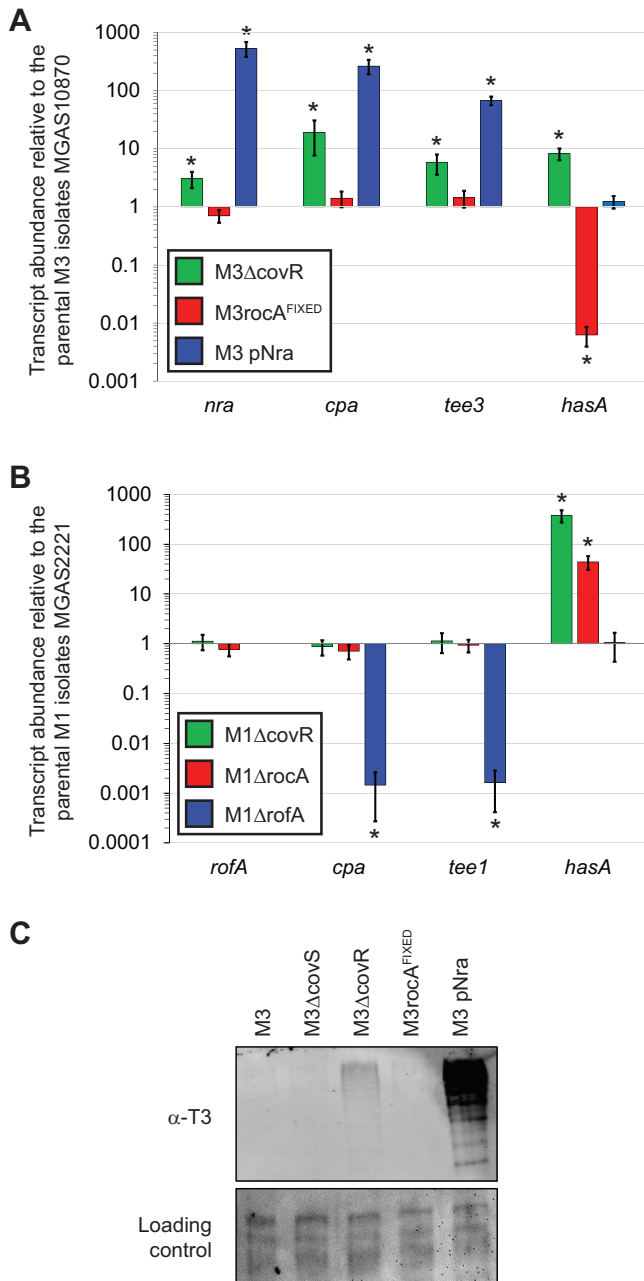
**Relative to pilus-expressing derivatives, clinical M3 GAS isolates display a reduced ability to adhere to host cells.** Given that in isolates of other serotypes pili have been shown to promote the adherence of GAS to host cells (24, 45, 46), we hypothesized that M3 isolates would have reduced adherence capabilities relative to pilus-producing derivatives. To test this hypothesis, we used derivatives of MGAS10870, M3Δ*nra*, and M3Δ*tee3* (a strain which does not produce pili due to the deletion of the *tee3* gene, encoding the major pilus protein) containing either the empty vector or p*Nra* in two tissue culture-based models of adherence. The data were similar for each of the models, which used VK2/E6E7 or HaCaT tissue culture cells, namely, that the expression of pili, due to the presence of p*Nra*, increased adherence ~2-fold over that for strains carrying only the empty vector (Fig. 7A and B). That this phenotype was a consequence of pilus expression was determined by the absence of any phenotype for p*Nra* in the strain M3Δ*tee3* background. Thus, through the reduction in the level of pilus expression, clinical serotype M3 GAS isolates have a reduced adherence phenotype.

**Clinical M3 GAS isolates have an increased ability to evade the innate immune system relative to pilus-expressing derivatives.** Published studies differ as to the influence of pili on the survival and proliferation of GAS in nonimmune whole human blood (25, 26, 33). A study with M1 GAS isolates identified that pili reduce survival (33), while studies with M2 and M53 GAS isolates identified that pili enhance survival (25, 26). To investigate this in the serotype M3 GAS background, we performed bactericidal assays. In agreement with the work performed in the M1 background (33), pilus expression through the presence of plasmid p*Nra* reduced survival rates ~2-fold in both the strain MGAS10870 and strain M3Δ*nra* backgrounds but not in the strain M3Δ*tee3* background (Fig. 8). Thus, the low level of pilus expression observed for clinical M3 isolates enhances their ability to survive in human blood and likely contributes to the association of serotype M3 isolates with particularly severe invasive infections.

## DISCUSSION

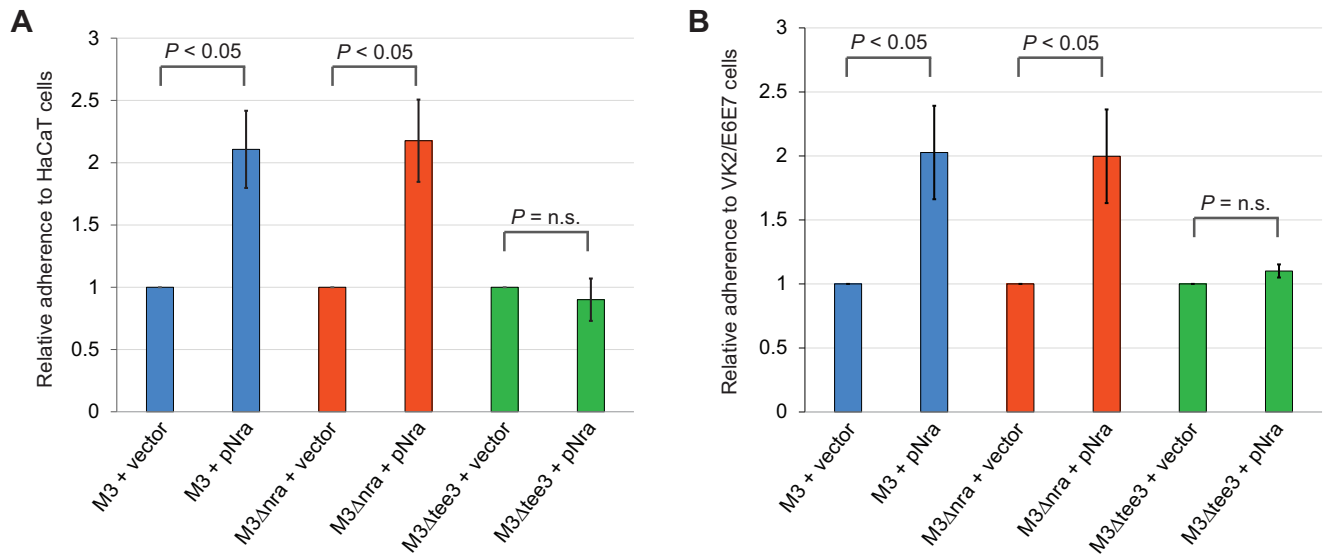
Bacterial intraspecies phenotypic variation can negatively impact public health by reducing the efficacy of diagnostic, treatment, and/or preventative measures (47–49). Thus, insights into the molecular mechanisms that drive this variation stand to benefit both basic science and clinical research. Here, we identified that GAS isolates show variation in the expression of pili on their cell surface. Specifically, unlike other GAS serotypes studied to date (e.g., serotypes M1, M2, M6, M28, M49, and M53), serotype M3 isolates produce no or very limited levels of pili, and hence, pilus expression occurs in





**FIG 6** CovR represses pilus expression in serotype M3, but not M1, GAS. (A) TaqMan-based quantitative RT-PCR. The parental M3 isolate MGAS10870 and derivatives M3ΔcovR, M3rocA<sup>FIXED</sup>, and M3 pNra were grown to the exponential phase before they were harvested and analyzed via quantitative RT-PCR. Shown are the averages ± standard deviations for triplicate biological samples run in duplicate. \*, statistically significant difference relative to the parental isolate (*t* test, *P* < 0.05). (B) As in panel A, except that parental M1 isolate MGAS2221 is compared to the derivatives M1ΔcovR, M1ΔrocA, and M1ΔrofA. (C) Western blot analysis. Cell wall proteins were isolated from the five indicated M3 GAS strains and analyzed by Western blotting with an anti-T3 antibody. The membrane was stained following protein transfer to serve as a loading control.

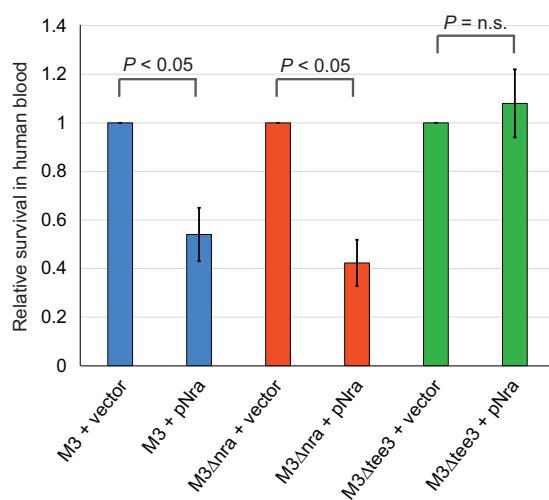
a serotype-specific manner. In part, this finding was based upon data from our interserotype transcriptome comparison, which are the first data from such a comparison to be reported. We also identified that the regulatory protein Nra is a positive regulator of pilus expression in M3 GAS isolates, while MsmR has no regulatory effect on pilus expression, and that the low level of pilus production by M3 GAS isolates correlates with low *nra* transcript levels. Finally, we discovered that the phenotypic



**FIG 7** Serotype M3 isolates have reduced rates of adherence as a consequence of low pilus expression. Tissue culture-based assay of adherence using HaCaT cells (a human keratinocyte line) (A) and VK2/E6E7 cells (a human epithelial cell line) (B). Derivatives of the parental M3 isolate MGAS10870, an *nra* deletion mutant (M3 $\Delta$ nra), and a *tee3* deletion mutant (M3 $\Delta$ tee3; which does not produce pili) containing either the empty vector or pNra were assessed for their ability to adhere to lawns of tissue culture cells. The percentage of the inoculum that adhered was determined, and the data are displayed as the adherence level relative to that of the empty-vector-containing strains. The experiments were performed in triplicate, with mean values  $\pm$  standard deviations being shown. Statistical significance was determined via the *t* test. n.s., not significant.

consequences of the low levels of pilus expression by M3 GAS isolates are a reduced ability to adhere to host cells and an increased ability to survive and proliferate in human blood.

We propose that the high level of M3 GAS survival in human blood, in part as a consequence of having reduced pilus expression, contributes to the association of M3 isolates with severe invasive infections. While a full explanation for why M3 isolates do not express significant levels of pili remains to be determined, our data are consistent with this being due to the attenuated transcription of *nra*, as *nra* transcript levels in M3



**FIG 8** Serotype M3 isolates have an enhanced ability to survive in human blood as a consequence of low levels of pilus expression. The results of the Lancefield bactericidal assay are shown. The same six isolates described in Fig. 7 were assessed for their ability to survive and replicate in human blood. The survival of each isolate was determined, and the data are presented as the survival level relative to that observed for the respective empty-vector-containing isolates. The experiment was performed in triplicate, with mean values  $\pm$  standard deviations being shown. Statistical significance was determined via the *t* test. n.s., not significant.

GAS are ~177-fold lower than *rofA* transcript levels in M1 GAS and 60-fold lower than *nra* transcript levels in M49 GAS (Fig. 3). Possible reasons for the low *nra* transcript levels in M3 GAS isolates include the following: (i) an M3 GAS-specific repressor protein negatively regulates *nra* transcription, (ii) *nra* mRNA could be highly unstable, or (iii) the *nra* promoter in M3 GAS may be functionally different (it may be weaker or have an additional repressor binding site) from that in pilus-expressing strains (note that only 8 SNPs differentiate the 430-bp *nra-cpa* intergenic regions in M3 and M49 GAS isolates, and none obviously influence promoter strength; see Fig. S3A in the supplemental material). Ultimately, regardless of the mechanism behind the low *nra* transcript abundance in M3 GAS, this represents a serotype-specific difference in gene regulation. Thus, along with mutations in the regulator-encoding genes *rivR*, *fasC*, and *rocA*, the reduction in *nra* transcription influences virulence factor expression in M3 GAS, producing a serotype-specific virulence factor expression profile that we propose underpins the association of M3 isolates with particularly severe and lethal invasive infections.

The Fas regulatory system consists of the putative sensor kinases FasB and FasC, the putative response regulatory FasA, and the small regulatory RNA FasX (50). All three Fas proteins are required for the significant production of FasX, and hence, FasX is essentially absent from serotype M3 isolates due to the natural *fasC* mutation (17). Where investigated (serotype M1, M2, M6, and M28 GAS isolates), FasX posttranscriptionally enhances the expression of streptokinase and represses the expression of the adhesins PrtF1 and PrtF2 and pili (51, 52). Thus, prior to the work presented here we had expected M3 isolates to show enhanced, not reduced, pilus expression relative to isolates of other serotypes. As pilus expression reduces GAS survival in human blood, at least in the serotype M1 and M3 backgrounds, it is possible that the apparent reduction in *nra* transcript levels in M3 GAS was selected for to combat the increase in pilus expression due to the *fasC* mutation, thus maintaining a substantial ability to survive and proliferate in blood. However, the order in which these genetic events (*fasC* mutation and *nra* transcript reduction) occur has yet to be determined.

The key finding of this research, that serotype M3 GAS isolates produce only negligible amounts of pili on their cell surface, was unexpected, given the findings presented in three previously published collections of articles. First, studies described in two publications biochemically characterized the ability of the M3 GAS pilus proteins to form pili (53, 54). Importantly, however, the findings from these studies were primarily gained using protein extracts from *Escherichia coli* strains overexpressing the M3 GAS pilus proteins, and thus, an analysis of whether M3 isolates themselves actually express pili was not performed. Second, pilus expression by M3 GAS was investigated in a study looking into the regulatory role of environmental pH, and similar to the findings for most other serotypes, the investigators identified that a low pH (pH 6.4) increased the level of M3 GAS pilus expression (55). However, while a lower pH increased the level of pilus expression, no pili were produced in the sample at neutral pH, consistent with our data. Note that the regulators responsible for this pH-specific regulation of pilus expression are unknown, but recent data indicate that the LiaFSR proteins may contribute (56, 57). Third, T typing is a serology-based method for dividing GAS isolates into different types (T types), and the major pilus protein (Tee) is the T antigen. A study by Johnson et al. typed more than 2,000 serotype M3 isolates and found that ~90% of them typed as T3 (the other 10% could not be typed) (58), which would indicate that most M3 isolates produce the Tee protein and therefore produce pili. However, several factors negatively affect the ability to T-type isolates; indeed, as stated by Johnson et al., "a strain with the very common T3 agglutination pattern may lose some, or even all, of the reactions making up that pattern depending on the intensity of trypsinization" (58). Trypsinization of the GAS protein samples prior to T typing is performed to reduce cross-reactivity to antibodies present in the T-typing sera that are not targeted toward the major pilus protein (pili are trypsin resistant) and, hence, would give false-positive results. The finding that extensive trypsinization can prevent the M3 GAS sample from typing as T3 would be consistent with the reacting

antibodies in the typing sera being targeted against nonpilus proteins, in addition to pilus proteins. Indeed, we observed extensive cross-reactivity when using the T3 typing sera in our Western blot analyses, even in strains, such as M3 $\Delta$ tee3, that do not produce pili. Consistent with this, we found that some T-typing sera do not harbor any antibodies against pilus proteins (data not shown). Thus, we hypothesize that M3 isolates type as T3 primarily due to the reactivity of antibodies against nonpilus proteins in the sera, which would be consistent with the data presented here and the observation that extensive trypsinization prevents T typing of M3 isolates.

Building upon previous work showing that CovR represses pilus expression in serotype M3 GAS (42), we generated data consistent with CovR regulating pilus expression indirectly by repressing *nra* transcription and thus the abundance of the *nra* transcript (Fig. 6A). While CovR appears to repress pilus expression in a serotype-specific manner (Fig. 6B), this regulation does not appear to be the major factor behind the negligible pilus expression of clinical M3 isolates, as evident by the still low level of pilus expression seen by strain M3 $\Delta$ covR (Fig. 6C). Why CovR represses pilus expression in M3, but not M1, GAS is unclear. However, the *nra-cpa* intergenic region in M3 GAS and the *rofA-cpa* intergenic region in M1 GAS are highly divergent (Fig. S3B). We propose that CovR binds only to the M3 intergenic region.

The nine characterized FCT types are variable in gene content, gene order, and gene sequence (28). Thus, it is not surprising that there are differences in the regulation and function of pili from different FCT types. In addition to those already described (e.g., the expression of *rofA* or *nra*, or the production of pili that increase or decrease GAS resistance to human blood), there are also differences in the functioning of individual pilus proteins. For example, a recent report of a study working in a serotype M2 background (FCT-6) identified that the pilus backbone protein Tee2 is the adhesive (collagen-binding) component (25). This is in contrast to pili from the FCT-2 and FCT-3 regions, in which the ancillary protein Cpa is the adhesive component (34, 38). Given the regulatory and functional differences between pili from different FCT types and their different contributions to GAS virulence, the further study of pili from the different FCT types is warranted.

Despite decades of research, no licensed GAS vaccine is currently available. Perhaps the most advanced effort to date, a multivalent vaccine encompassing multiple M protein hypervariable regions, has yielded promising preliminary results (59). However, while an M protein-based vaccine may be of value, the large diversity of M types in the population prevents this approach from producing a universally protective vaccine. It has been identified that Tee, the pilus backbone protein, provides antigenic protection in murine models of infection (22). Thus, development of a vaccine based upon the targeting of pilus proteins, by themselves or in combination with other GAS proteins, is an area of interest (60, 61). While this approach, like the M protein-based approach, also suffers from differences in antigen sequence across GAS isolates, there is less complexity in the population (62). A potential problem unique to the creation of a pilus-based vaccine, however, is highlighted by the data generated in this study, namely, that isolates of at least one GAS serotype (serotype M3) do not express appreciable levels of pili on their cell surface, bringing into question the efficacy of such a vaccine against infections caused by this serotype. Thus, our data may inform vaccine development, and we are currently investigating whether a phenotype of low levels of pilus expression is shared by additional prevalent GAS serotypes.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The representative serotype M1 and M3 clinical GAS isolates MGAS2221 and MGAS10870, respectively, were the main strains used in this study. Additional GAS isolates and derivatives of the clinical isolates that we created are listed in Table S2 in the supplemental material. GAS isolates were grown in Todd-Hewitt broth containing 0.2% yeast extract (THY broth). Chloramphenicol (4  $\mu$ g/ml), kanamycin (300  $\mu$ g/ml), or spectinomycin (150  $\mu$ g/ml) was added to the THY broth when needed.

**Total RNA isolation and RNA-Seq analysis.** Total RNA was isolated as previously described (9). Briefly, triplicate cultures of the parental serotype M1 strain MGAS2221 and the parental serotype M3 GAS strain MGAS10870 were grown to the exponential phase of growth (corresponding to an optical

density at 600 nm [OD<sub>600</sub>] of 0.5) in THY broth. One volume of GAS culture was subsequently added to 2 volumes of RNAprotect Bacteria reagent (Qiagen) and incubated at room temperature for 5 min. Following centrifugation at 5,000 × *g* for 10 min at 4°C, the bacterial pellets were snap-frozen in liquid nitrogen and stored at −80°C until they were ready for use. Bacterial cells were processed for RNA isolation using a mechanical lysis method in conjunction with an RNeasy minikit (Qiagen). Contaminating DNA was removed with three 40-min treatments with the Turbo DNase-free reagent (Life Technologies). The quality and quantity of the isolated RNA were assessed by using a Bioanalyzer 2100 system (Agilent Technologies). To process the RNA for RNA-Seq analysis, rRNAs were depleted using a Ribo-Zero gram-positive rRNA removal kit (Epicentre). The rRNA-depleted RNA was then used to generate cDNA libraries using a ScriptSeq kit (Epicentre) (63). To do this, RNA was fragmented, cDNA was synthesized using random hexamers containing a 5′ tagging sequence, the RNA was hydrolyzed, and the cDNA was tagged at the 3′ end. A limited number of PCR cycles (*n* = 12) was used to amplify the libraries via the 5′ and 3′ tags (the libraries were barcoded by using different primers), and the libraries were selected by size (170 to 300 bp). The size-selected and barcoded libraries were run on an Illumina MiSeq flow cell. Data were analyzed using the bioinformatic program Rockhopper (63). The M1 GAS data files were run against the MGAS5005 (a fully sequenced M1 GAS isolate [64]) genome sequence, while the M3 GAS data files were run against the MGAS315 (a fully sequenced M3 GAS isolate [39]) genome sequence. Sequencing data from different experiments were normalized using upper-quartile normalization (65). Orthologous genes between the two data sets were identified and tested for differential expression using the approach of DESeq (66), with correction for multiple tests (67).

**Absolute quantification assays.** In order to compare the baseline levels of the expression of the transcripts encoding the regulatory proteins RofA and Nra, as well as those encoding the major (*tee*) and minor (*cpa*) pilus proteins, in M1, M3, and M49 GAS isolates, we performed a TaqMan-based absolute quantification assay. Ten M1, 19 M3, and 8 M49 clinical GAS isolates, recovered in a temporally and spatially diverse manner (Table S2), were grown in duplicate to mid-exponential phase (OD<sub>600</sub> 0.5), total RNA was collected and treated twice with DNase (Turbo DNase; Ambion), and its integrity and concentration were evaluated with a Bioanalyzer instrument (model 2100; Agilent). cDNA was synthesized using a SuperScript III kit (Invitrogen) and analyzed by quantitative RT-PCR using a CFX Connect real-time system (Bio-Rad). Specifically, we compared the expression of target genes in these strains against a standard curve generated using 10-fold dilutions of MGAS2221 (M1), MGAS10870 (M3), or 591 (M49) genomic DNA, normalized to the genome size of the respective strains. The TaqMan primers and probes used for this assay are listed in Table S3.

**Creation of plasmids pNra, pMsmR, pNra-M3, pNra-M3<sup>FIX</sup>, and pNra-M18.** Plasmids pNra, pNra-M3, and pNra-M18 were created by amplifying the *nra* genes (via primers UNR443 and UNR444; Table S3) from M49, M3, and M18 GAS genomes, respectively, and cloning them into the shuttle vector pDCBB, which is a derivative of pDC123 (68). Plasmid pMsmR was created by amplifying the *msmR* gene (via primers UNR441 and UNR442) from M49 GAS and cloning it into the vector pDCBB. Plasmid pNra-M3<sup>FIX</sup> was created by amplifying the *nra* gene from GAS strain M3nra<sup>FIXED</sup> using primers UNR443 and UNR444. The sequences of all plasmids were verified.

**Creation of M3 GAS derivatives M3Δnra, M3ΔmsmR, M3nra<sup>FIXED</sup>, M3nra<sup>FIXED</sup>ΔmsmR, M3.VNTR, M3ΔcovR, and M3ΔcovS.** To facilitate the investigation of pilus expression in serotype M3 GAS isolates, we created derivatives of the representative M3 isolate MGAS10870. The PCR primers used to make these strains are listed in Table S3. Note that all constructed GAS strains were subjected to targeted PCR and sequencing to ensure that the expected DNA change(s) had occurred. Strains M3Δnra and M3ΔmsmR were created by replacing the *nra* and *msmR* genes, respectively, with a spectinomycin resistance cassette. Flanking sequences (~1 kb in size) upstream and downstream of each gene were joined by overlap extension PCR to either side of the spectinomycin resistance cassette (1 kb), and the sequences were cloned into pCR2.1-TOPO (Thermo Fisher). After sequence verification, the 3-kb insert was PCR amplified and used to transform MGAS10870 competent cells, selecting on THY agar containing spectinomycin. Strain M3nra<sup>FIXED</sup> was created by an allelic exchange method, using the suicide vector pBBL740, similar to our previously described method (7). Briefly, primers UNR222/UNR224 and UNR96/UNR223 were used to amplify 1-kb regions of *nra* such that, when joined together via Gibson assembly, they generate a 2-kb product that contains the required DNA change. This product was cloned into pBBL740, also via Gibson assembly, and the sequence was verified. The plasmid was subsequently transformed into MGAS10870 competent cells, selecting on THY agar containing chloramphenicol (to ensure plasmid integration into the GAS chromosome). Transformants were passaged in THY broth (no antibiotics) to allow the excision of the plasmid out of the chromosome, and chloramphenicol-sensitive colonies were identified by patch plating onto both THY agar and THY agar containing chloramphenicol. Sensitive strains were subjected to targeted PCR and sequencing to identify whether they contained the parental or modified *nra* allele. One strain that harbored the modified allele was retained and named M3nra<sup>FIXED</sup>. Strain M3nra<sup>FIXED</sup>ΔmsmR is a derivative of M3nra<sup>FIXED</sup> in which *msmR* was replaced with the spectinomycin resistance cassette, as described above for strain M3ΔmsmR. Strain M3.VNTR was created by an allelic exchange method, using suicide vector pBBL740, similar to that described above for strain M3nra<sup>FIXED</sup>. The primers used to create the plasmid insert in this case were UNR619/UNR623 and UNR620/UNR625. Strain M3ΔcovS, which contains a 2-bp deletion within *covS*, was also created by allelic exchange, with the plasmid insert being created via overlap extension PCR using the PCR products generated with primers BBLCS1/BBLCS4 and BBLCS2/BBLCS3. Strain M3ΔcovR was created by replacement of an internal fragment of *covR* with a promoterless *aphA3* gene encoding kanamycin resistance (amplified from strain JRS950 via primers COVUPUP and COVSR1) (69).

**Isolation of cell wall proteins.** Bacterial cultures for all strains were grown to mid-exponential phase ( $OD_{600}$ , 0.5) in THY broth. Forty-five-milliliter aliquots were recovered, and the bacteria were pelleted by centrifugation ( $5,000 \times g$ , 11 min). The bacterial pellets were washed once in 10 ml of TE (Tris-EDTA) buffer and then resuspended in 900  $\mu$ l of TE-sucrose buffer (50 mM Tris-HCl, pH 7.5, 550 mM sucrose, 1 mM EDTA, 250  $\mu$ g ml<sup>-1</sup> hyaluronidase, 1 mg ml<sup>-1</sup> lysozyme, 250  $\mu$ g ml<sup>-1</sup> mutanolysin). Samples were incubated at 37°C with end-over-end rotation for 2 h and then centrifuged at  $15,000 \times g$  for 5 min in order to pellet the protoplasts. The supernatants, containing cell wall proteins, were removed to clean 1.5-ml tubes, and the centrifugation step was repeated. The final supernatants were buffered 1:1 with  $2 \times$  Laemmli buffer and stored at  $-20^\circ\text{C}$  until analysis.

**Western blot analysis of cell wall proteins.** The cell wall protein fractions were separated on a 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane, and the membrane was stained for use as a loading control using a MemCode reversible protein stain kit (Pierce). The same membrane was then subjected to overnight incubation with an anti-T3 monoclonal antibody (1:1,000 dilution; TransEurope Chemicals). The next day, the blot was washed thoroughly and then incubated with anti-rabbit IgG secondary antibody labeled with Alexa Fluor 680, and a Li-Cor Odyssey system was used to visualize and capture the fluorescent signal.

**TaqMan-based quantitative RT-PCR analysis.** Total RNA was isolated for quantitative RT-PCR analysis of the pilus transcripts. All cultures were grown to mid-exponential phase (corresponding to an  $OD_{600}$  of 0.5), mechanically disrupted, and harvested for RNA using an RNeasy minikit (Qiagen). cDNA was synthesized using an Invitrogen SuperScript kit per the manufacturer's protocols. Through utilization of the CFX Connect real-time system (Bio-Rad), RT-PCR analysis was conducted with the primers and probes listed in Table S3.

**Tissue culture adherence assay.** Cells of the human epithelial cell line VK2/E6E7 were cultured using keratinocyte serum-free medium (KSF; Gibco) containing 0.1 ng/ml human recombinant epidermal growth factor 1-53 (EGF 1-53) and 0.05 mg/ml bovine pituitary extract (BPE), calcium chloride at 44.1 mg/liter (final concentration, 0.4 mM), 50  $\mu$ g/ml streptomycin, and 50 units/ml of penicillin. Upon nearing confluence, cells were resuspended in KSF medium without antibiotics, seeded into 12 wells of a 12-well tissue culture plate, and then incubated for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere. Cells of the HaCaT (immortalized human keratinocyte) cell line were cultured using Dulbecco modified Eagle medium (DMEM; ATCC) containing 10% fetal bovine serum, 50  $\mu$ g/ml streptomycin, and 50 units/ml of penicillin. Upon nearing confluence, the cells were resuspended in DMEM without antibiotics, seeded into 12 wells of a 12-well tissue culture plate, and then incubated for 24 h at 37°C in a 5% CO<sub>2</sub> atmosphere. To prepare GAS isolates for this assay, the strains were grown to an  $OD_{600}$  of 0.4, 1 ml of culture was pelleted, and the cell pellets were resuspended in 1 ml of phosphate-buffered saline (PBS). One hundred microliters of each GAS solution was added to separate wells of the seeded 12-well tissue culture plates (VK2/E6E7 or HaCaT cells; resulting in a multiplicity of infection of  $\sim 50$ ). At the same time, 100  $\mu$ l of the PBS-diluted GAS cultures (1:1,000,000) was plated onto blood agar plates to enable accurate calculation of the initial inocula. The plate was incubated for 5 min (37°C in 5% CO<sub>2</sub>). The liquid in the wells was removed, and the wells were washed five times with 1 ml of PBS. The tissue culture cells were lysed by incubating them for 15 min in 1% saponin in PBS at room temperature. The lysed tissue culture cells and adhered GAS cells were scraped from the bottom of each well. The lysate was serially diluted (1:100 and 1:1,000), and 100  $\mu$ l of each dilution was plated onto blood agar plates in duplicate. The average number of bacteria was determined, and the percentage of adhering bacteria relative to the size of the inoculum was calculated.

**Lancefield bactericidal assay.** To test the ability of individual GAS strains to survive in human blood, we performed Lancefield bactericidal assays. Cultures of each strain were grown to early exponential phase ( $OD_{600}$ , 0.15 to 0.20). Each GAS culture was diluted to  $10^{-4}$  in sterile phosphate-buffered saline, and 450  $\mu$ l of whole heparinized blood was added to 50  $\mu$ l dilute culture. These mixtures were then incubated for 3 h at 37°C with end-over-end rotation. Fifty microliters of each inoculum was simultaneously plated on blood agar plates to allow enumeration on the next day. Following incubation, the GAS-blood cultures were diluted and plated on blood agar plates. All samples were incubated overnight at 37°C in a 5% CO<sub>2</sub> atmosphere. The number of CFU was calculated, and the data are presented as the rate of survival relative to that of the empty-vector-containing strains after performing the calculation [(number of surviving CFU/initial number of CFU)  $\times 100$ ].

**Accession number(s).** The RNA-Seq data have been deposited at the Gene Expression Omnibus (GEO) database at the National Center for Biotechnology Information and are accessible through accession number [GSE97935](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97935).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00792-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 1.9 MB.

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