Virulence of serotype M3 Group A Streptococcus strains in wax worms (Galleria mellonella larvae)

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Key words: Group A Streptococcus, host-pathogen interactions, Galleria mellonella larvae, invasive infection, invertebrate model

Group A Streptococcus (GAS) causes human infections that range in severity from pharyngitis (“strep-throat”) to necrotizing fasciitis (“flesh-eating disease”). To facilitate investigation of the molecular basis of host-pathogen interactions, infection models capable of rapidly screening for differences in GAS strain virulence are needed. To this end, we developed a Galleria mellonella larva (wax worm) model of invasive GAS infection and used it to compare the virulence of serotype M3 GAS strains. We found that GAS causes severe tissue damage and kills wax worms in a dose-dependent manner. The virulence of genetically distinct GAS strains was compared by Kaplan-Meier survival analysis and determining 50% lethal doses (LD₅₀). Host-pathogen interactions were further characterized using quantitative culture, histopathology and TaqMan assays. GAS strains known to be highly pathogenic in mice and monkeys caused significantly lower survival and had significantly lower LD₅₀ in wax worms than GAS strains associated with attenuated virulence or asymptomatic carriage. Furthermore, isogenic inactivation of proven virulence factors resulted in a significantly increased LD₅₀ and decreased lesion size compared to the wild-type strain, a finding that also strongly correlates with animal studies. Importantly, survival analysis and LD₅₀ determination in wax worms supported our hypothesis that a newly emerged GAS subclone that is epidemiologically associated with more human necrotizing fasciitis cases than its progenitor lineage has significantly increased virulence. We conclude that GAS virulence in wax worms strongly correlates with the data obtained in vertebrate models, and thus, the Galleria mellonella larva is a useful host organism to study GAS pathogenesis.

Introduction

Advances in next-generation DNA sequencing technology and bioinformatics tools now make it possible to efficiently and economically perform genome-wide association studies on large bacterial strain collections. This to end, our laboratory has extensively used a comparative pathogenomic strategy to investigate the molecular genetic relationships between strain genotypes and disease phenotypes in human patients infected with serotype M3 strains of Group A Streptococcus (GAS, Streptococcus pyogenes). GAS is a human pathogen that causes infections ranging in severity from uncomplicated pharyngitis (“strep-throat”) and impetigo to life-threatening necrotizing fasciitis (“flesh-eating disease”) and necrotizing pneumonia. Serotype M3 strains of GAS are particularly interesting because they demonstrate epidemic behavior, undergo rapid shifts in disease frequency, and cause a disproportionate number of invasive and lethal infections compared to many other GAS serotypes.

Our study of GAS strain genotype-human disease phenotype relationships has been made possible by the availability of a comprehensive 18 year prospective population-based sample of serotype M3 GAS strains collected from patients with invasive infections in Ontario, Canada. To date, whole genome sequences have been determined for ~100 serotype M3 GAS strains collected from well-described clinical episodes, resulting in new insight to the emergence of subclone populations and the evolution of strain virulence. For example, we recently discovered the significant role of the mtsR-prsA-SpeB virulence axis to GAS necrotizing fasciitis capacity. These studies have relied heavily on the testing of new hypotheses in mouse and monkey models; however, to keep pace with this quickly progressing line of investigation, new models capable of rapidly screening strain virulence are needed. To this end, we sought to develop a Galleria mellonella larva model of invasive GAS infection.

Larvae of the greater wax moth (Galleria mellonella), colloquially termed “wax worms” due to their natural lifestyle of infesting beehives and consuming beeswax, have been recently used as an alternative to vertebrates as a model host for studying pathogenic microbes. These include studies with Acinetobacter baumannii, Aspergillus spp., Bacillus cereus, Burkholderia cepacia, Candida albicans, Cryptococcus neoformans, Francisella tularensis, Listeria spp. and Pseudomonas aeruginosa. Wax worms have also been used to study the virulence of Staphylococcus aureus, a Gram-positive bacterium that has many overlapping themes with GAS in the mechanism of its virulence factors. Both organisms effectively evade host
Furthermore, at temperatures either above or below human body temperature.27 GAS transcriptome markedly changes when cultures are grown as pathogens, and in contrast to other invertebrate model hosts such as Caenorhabditis elegans or Drosophila melanogaster, wax worms can be maintained at 37°C.15 This trait is important because the GAS transcriptome markedly changes when cultures are grown at temperatures either above or below human body temperature.27 Furthermore, Galleria mellonella larvae have an immune system with reasonable homology to vertebrates.15 The hemocoel contains a digestive tract, loosely organized muscular system, biosynthetic fat body and hemolymph. These tissue types are similar to those encountered by GAS during invasive infections in humans. The hemolymph is analogous to blood in that it transports nutrients, hemocytes and immune molecules. At least two of the six subsets of hemocytes described in Galleria mellonella larvae are capable of phagocytosis.28 Also, numerous enzymatic cascades akin to complement fixation and blood coagulation occur in the hemolymph.15 These complex multi-component reactions result in hemolymph clotting and melanin production, key defense mechanisms against invading microbes.29 As such, we hypothesized that the Galleria mellonella larva is a suitable host organism to study GAS pathogenesis. Herein, we describe studies designed to develop a Galleria mellonella larvae model of invasive GAS infection. This new model will be particularly useful as a rapid bioassay for screening differences in virulence among GAS isolates in large strain collections.

Figure 1. GAS causes severe disseminated infection and tissue destruction in Galleria mellonella larvae. (A) Wax worm larvae were inoculated with 10⁷ CFU of representative serotype M3 strain MGAS315 by injection through the left hindmost proleg using a 29 G needle. (B and C) Infection with MGAS315 resulted in rapid melanization (dark pigmentation) and high mortality (n = 10 larvae, red bar) by 24 h post-inoculation. No deaths occurred in larvae sham inoculated with sterile saline (PBS, gray bar). (D) Microscopic examination of infected wax worm larvae at 6 h post-inoculation shows an expanding abscess-like lesion at the GAS injection site. An area of extensive soft tissue necrosis and bacterial proliferation (dense blue-violet region in the center of the micrograph, demarcated with a white asterisk) is surrounded by melanized hemocytes (extracellular brown pigment and host immune cells walling off the expanding lesion, demarcated by black arrowheads). Extensive tissue destruction occurred in the thoracic and abdominal body segments (Gram’s stain, 4x original magnification). (E) At the site of inoculation, a dense infiltrate of GAS organisms is seen in a background of anucleate agranular host hemocytes (Gram’s stain, 40x original magnification). (F) By 6 h post-inoculation, chains of GAS have already disseminated throughout the hemolymph to begin infecting soft tissue near the first true leg (boxed region in A). The Galleria mellonella body cavity contains a variety of soft tissue types, including hemolymph with immune cells, fat, muscle and epithelium, that are similar to those encountered by GAS during invasive human infections such as necrotizing fasciitis (Gram’s stain, 40x original magnification).

Results

GAS causes severe tissue destruction and disseminated infection in Galleria mellonella larvae. GAS is a host-specific pathogen, causing natural disease only in humans. Several known and putative GAS virulence factors have only modest activity against their target molecules in other species.30-32 Thus, mouse and lower vertebrate infection models may have a somewhat limited capacity to test particular hypotheses bearing on GAS virulence. To begin to test the hypothesis that the Galleria mellonella larva is a suitable model host to study GAS pathogenesis, wax worms were infected with 10⁹ CFU of representative serotype M3 strain MGAS315 and examined by visual and microscopic analysis (Fig. 1A). This strain was selected because its genome has been sequenced, it is representative of highly virulent serotype M3 GAS strains causing severe invasive disease in humans, and it has been extensively studied in previous molecular pathogenesis experiments using mice and monkeys.13,14,33,34 Wax worm larvae that were sham-inoculated with sterile saline had no change in their appearance or activity. In contrast, all larvae infected with strain MGAS315 had distinct signs of invasive infection, including melanization (Fig. 1B), rapid death (Fig. 1C) and formation of a destructive abscess-like lesion at the site of inoculation (Fig. 1D). These abscesses were comprised of a dense central core of necrotic tissue and GAS organisms surrounded by a well-organized outer band of host hemocytes, coagulated hemolymph and extracellular melanin pigment (Fig. 1D). Most GAS present at the inoculation site were extracellular, suggesting they had overwhelmed the capacity of host phagocytes to contain the infection (Fig. 1E). Also, many GAS organisms escaped from the melanin clot encapsulating the abscess to disseminate throughout the hemocoel (Fig. 1F). These findings are similar to the histopathology that is commonly observed in mouse and monkey models of GAS necrotizing fasciitis and in humans with severe soft tissue infections. Thus, these results support the hypothesis that the Galleria mellonella larva is a suitable model host for studying GAS pathogenesis.
GAS causes dose-dependent killing in *Galleria mellonella* larvae. To test the hypothesis that *Galleria mellonella* larvae are susceptible to dose-dependent killing by GAS, wax worms were inoculated with serial 10-fold dilutions of the highly virulent reference strain MGAS315, and survival was monitored for 96 h. Similarly, larvae were also inoculated with the same dilutions of strain MGAS12501, a GAS strain that was recovered from the oropharynx of an asymptomatic carrier. Survival was monitored for 96 h. Kaplan-Meier survival curves were determined for four or more independent experiments and showed a dose-dependent response with both strains (n = 10 larvae per dose per strain per experiment). The highest dose (10^7 CFU) of either strain resulted in significantly higher mortality than any of the three lower doses tested (p < 0.001 for either MGAS315 or MGAS12501, 10^6 CFU compared to 10^7 CFU, logrank test). Similarly, inoculation of 10^7 CFU resulted in significantly higher mortality than the lowest dose tested (p < 0.001 for MGAS315 and p = 0.033 for MGAS12501, 10^4 CFU compared to 10^7 CFU, logrank test). At the 10^6 CFU dose, invasive strain MGAS315 killed more wax worms than carrier strain MGAS12501 (p = 0.004, logrank test). No deaths occurred in larvae sham infected with sterile saline (PBS, gray circles). (C–D) Survival curves of wax worm larvae inoculated with either invasive strain MGAS315 (red squares) or carrier strain MGAS12501 (brown triangles) were highly reproducible. Results from multiple independent experiments using the highest dose tested (10^7, solid lines) and lowest dose tested (10^4 CFU, dotted lines) are shown as individual curves (n = 10 larvae per dose per strain per experiment). (E) Infected larvae demonstrated progressive melanization post-inoculation, indicating a robust host immune response to the invasive GAS infection. Compared to larvae infected with the highly virulent invasive strain MGAS315, wax worms infected with the less virulent carrier strain MGAS12501 developed pigmentation at a much slower rate. (F) In vivo growth of GAS organisms in wax worm larvae inoculated with 10^6 CFU of strain MGAS315 demonstrates a logarithmic curve. Bacterial burden was quantified from pools of five homogenized larvae at the time points indicated (n = 3 pools per time point).

**Figure 2.** Virulence of GAS in the *Galleria mellonella* infection model. (A and B) Wax worm larvae were inoculated with serial dilutions (10^7, 10^6, 10^5 and 10^4 CFU) of strain MGAS315 (red squares), which is representative of highly virulent serotype M3 GAS strains causing severe invasive infections in humans or strain MGAS12501 (brown triangles), which was recovered from the oropharynx of an asymptomatic carrier. Survival was monitored for 96 h. Kaplan-Meier survival curves were determined for four or more independent experiments and showed a dose-dependent response with both strains (n = 10 larvae per dose per strain per experiment). The highest dose (10^7 CFU) of either strain resulted in significantly higher mortality than any of the three lower doses tested (p < 0.001 for either MGAS315 or MGAS12501, 10^6 CFU compared to 10^7 CFU, logrank test). Similarly, inoculation of 10^7 CFU resulted in significantly higher mortality than the lowest dose tested (p < 0.001 for MGAS315 and p = 0.033 for MGAS12501, 10^4 CFU compared to 10^7 CFU, logrank test). At the 10^6 CFU dose, invasive strain MGAS315 killed more wax worms than carrier strain MGAS12501 (p = 0.004, logrank test). No deaths occurred in larvae sham infected with sterile saline (PBS, gray circles). (C–D) Survival curves of wax worm larvae inoculated with either invasive strain MGAS315 (red squares) or carrier strain MGAS12501 (brown triangles) were highly reproducible. Results from multiple independent experiments using the highest dose tested (10^7, solid lines) and lowest dose tested (10^4 CFU, dotted lines) are shown as individual curves (n = 10 larvae per dose per strain per experiment). (E) Infected larvae demonstrated progressive melanization post-inoculation, indicating a robust host immune response to the invasive GAS infection. Compared to larvae infected with the highly virulent invasive strain MGAS315, wax worms infected with the less virulent carrier strain MGAS12501 developed pigmentation at a much slower rate. (F) In vivo growth of GAS organisms in wax worm larvae inoculated with 10^6 CFU of strain MGAS315 demonstrates a logarithmic curve. Bacterial burden was quantified from pools of five homogenized larvae at the time points indicated (n = 3 pools per time point).
strain in wax worms. Compared to the highly virulent reference strain MGAS315, which causes severe tissue destruction and high mortality in mice and monkeys, each GAS strain tested could be categorized as having either similar high or significantly less virulence capacity in wax worms (Fig. 3C). Importantly, the LD\textsubscript{50} of each GAS strain tested in wax worms had a near-linear relationship with its LD\textsubscript{50} in mice (Fig. 3D). Taken together, these findings are consistent with the hypothesis that GAS strain virulence in Galleria mellonella larvae correlates with virulence in mice.

Virulence of isogenic mutant GAS strains in Galleria mellonella larvae correlates with virulence in mice. We previously characterized the virulence of several isogenic mutant strains of GAS in mice and monkeys (Flores AR, Musser JM, unpublished data).\textsuperscript{13,34} These mutant strains of MGAS315 are deficient in the expression of the genes encoding metal transporter of Streptococcus regulator (mtsR), multiple gene activator of Streptococcus (mga), peptidyl-prolyl cis-trans isomerase A (prsA), streptococcal phospholipase A2 (slaA) or secreted streptococcal cysteine protease B (speB). Each isogenic mutant strain has significantly reduced virulence in models of necrotizing fasciitis, bacteremia and soft tissue infection (Flores AR, Musser JM, unpublished data).\textsuperscript{13,34} To further test the hypothesis that GAS strain virulence in Galleria mellonella larvae correlates with virulence in commonly used vertebrate models, Kaplan-Meier survival curves (Fig. 4A) and the 50\% lethal dose (LD\textsubscript{50}) (Fig. 4B) of each isogenic mutant strain were determined in wax worms. Compared to highly virulent reference strain MGAS315, the LD\textsubscript{50} of all five gene-deficient strains was significantly higher, that is, they were significantly less virulent in wax worm larvae (Fig. 4B). Wild-type virulence capacity was restored by gene complementation in all three strains tested (Fig. 4B).

One potential explanation for the altered virulence capacity of GAS strains deficient in these virulence factors is decreased replication or survival in vivo. To test this hypothesis, GAS burden was measured in wax worm larvae infected with isogenic mutant strains. However, consistent with previous data from mouse and monkey models,\textsuperscript{13} there was no significant difference in the CFUs recovered between the parental wild-type strain MGAS315 and the isogenic mutant strains (Fig. 4C). An alternative hypothesis for the altered virulence capacity of the isogenic mutant GAS strains is a decreased ability to destroy host tissue in vivo. To test this hypothesis, tissue histopathology was examined in infected larvae. Consistent with this hypothesis, wild-type strain MGAS315 caused significantly larger lesions with more melanization than the less virulent gene-deficient strains (Fig. 4D).

\textbf{Figure 3.} Virulence of serotype M3 GAS strains in the Galleria mellonella infection model correlates with virulence in mice. Wax worm larvae were infected with serial dilutions (10\(^7\), 10\(^6\), 10\(^5\) and 10\(^4\) CFU) of representative serotype M3 strain MGAS315 or one of 10 other GAS strains also recovered from human patients, and survival was monitored for 96 h. Results from three or more independent assays are shown (n = 10 larvae per strain per dose per experiment). (A) Kaplan-Meier survival curves were determined for wax worms inoculated with 10\(^{5}\) CFU. Compared to all strains tested except MGAS3392 (pink circles), the highly virulent reference strain MGAS315 (red circles) results in a significantly lower survival over time (“indicates p < 0.05 compared to MGAS315, logrank test). (B) The 50\% lethal dose (LD\textsubscript{50}) was determined by Probit analysis. The highly virulent reference strain MGAS315 (red bar) has a significantly lower LD\textsubscript{50} (that is, it is more virulent) than other invasive strains known to be less virulent in mice (various blue bars) or strains recovered from the oropharynx of asymptomatic carriers (various brown bars) (“indicates p < 0.05 compared to MGAS315, Mann-Whitney test). (C) Overall, GAS strains such as MGAS315, MGAS3382 and MGAS3392 which are known to be highly virulent in mice and/or monkeys (red checked bar) have a significantly lower mean LD\textsubscript{50} in wax worm larvae (that is, they are more virulent) than GAS strains associated with lower virulence in animals (blue checked bar) or asymptomatic carriage in humans (brown checked bar) (“indicates p < 0.01 compared to MGAS315, Mann-Whitney test). (D) The virulence of these 11 serotype M3 GAS strains tested in Galleria mellonella larvae strongly correlates with their virulence in mice (Pearson’s correlation \(r = 0.689\) and p = 0.019).
The mtsR-prsA-SpeB virulence axis is crucial to necrotizing fasciitis capacity in mice, monkeys and humans.15 To confirm that these genes are also expressed in vivo in *Galleria mellonella* larvae infected with reference wild-type strain MGAS315, TaqMan real-time quantitative PCR assays were performed on hemolymph collected 18 h post-inoculation. Relative to the highly expressed reference gene *tufA*, transcripts for *mtsR* and *prsA* were abundant (Fig. 4E). Consistent with recently published data bearing on the central role of SpeB-mediated tissue destruction in severe invasive infections,13,35,36 *speB* transcripts were highly abundant in the infected tissue (Fig. 4E).

Subclone 8 serotype M3 GAS strains are more virulent than subclone 5 strains in *Galleria mellonella* larvae. We previously reported that a clonally related subset of serotype M3 GAS invasive infection isolates, designated subclone 5 strains, are epidemiologically associated with decreased necrotizing fasciitis cases in humans and have a significantly decreased capacity to cause necrotizing fasciitis in mice.5,12 Ongoing epidemiological surveillance studies and genome sequencing have recently identified a new GAS strain lineage, designated subclone 8 strains, that are genetically descended from the necrotizing fasciitis-negative subclone 5 strains.5 Importantly, subclone 8 strains have apparently regained necrotizing fasciitis capacity in humans.5 However, this epidemiological association has not been investigated in animal models. To test the hypothesis that subclone 8 strains have increased virulence compared to their parental subclone 5 strains, Kaplan-Meier survival curves and the 50% lethal dose (LD50) of five representative strains from each subclone lineage were determined in wax worms. Results demonstrated that compared to subclone 5 strains, subclone 8 strains caused significantly lower survival over time (Fig. 5A) and have a significantly lower mean LD50 (Fig. 5B). That is, subclone 8 strains are significantly more virulent than subclone 5 strains in wax worms. This finding is consistent with the human epidemiological data and suggests that further investigation of the molecular mechanism responsible for restoring necrotizing fasciitis capacity to subclone 8 strains is warranted.

**Discussion**

GAS is a human-specific pathogen with no known animal reservoir. As a consequence, we have extensively used non-human primates, the most human-relevant animal model possible, as the preferred host for testing hypotheses bearing on GAS molecular pathogenesis.13,14,34,37,39 However, high cost and substantial
mice, and results of our recent investigation into the lence factor was not fully appreciated until studied in humanized colonic tissues for TaqMan or expression microarray analyses. This helps to overcome the challenges associated with extracting a sufficient quantity of high quality GAS RNA from infected animal tissues for TaqMan or expression microarray analyses.

Figure 5. Subclone 8 GAS strains are significantly more virulent than subclone 5 GAS strains in the Galleria mellonella infection model. Subclone 5 strains of GAS were previously shown to lack necrotizing fasciitis capacity in mice, monkeys and humans. Subclone 8 strains of GAS are newly emerged descendents of subclone 5 strains that epidemiologically have regained necrotizing fasciitis capacity in humans. To test the hypothesis that subclone 8 strains are more virulent than subclone 5 strains, wax worm larvae were infected with serial dilutions (10^7, 10^6, 10^5 and 10^4 CFU) of five representative strains from each subclone lineage, and survival was monitored for 96 h. Results from five independent assays are shown (n = 10 larvae per strain per dose per experiment with five strains tested from each subclone lineage). (A) Kaplan-Meier survival curves were determined for wax worms inoculated with 10^7 CFU. Compared to subclone 5 strains (violet diamonds), subclone 8 strains (pink diamonds) result in significantly lower survival over time (logrank test). (B) The 50% lethal dose (LD_50) was determined by Probit analysis. Subclone 8 strains (pink bars) have a significantly lower LD_50 (that is, they are more virulent) than subclone 5 strains (violet bars) (Mann-Whitney test).

experimental time may limit the use of monkeys in GAS research. Mice are also commonly used, but murine models suffer from some of the same limitations, plus they may be further hindered by a poor recapitulation of the complex host-pathogen interactions that underlie human infectious disease phenotypes. For example, the importance of streptokinase (ska) as a GAS virulence factor was not fully appreciated until studied in humanized mice, and results of our recent investigation into the mtsR-prsA-SpeB virulence axis were confirmed in monkeys. For this reason, any new GAS-host model must be thoroughly validated to appropriately interpret virulence assessments. As such, we carefully designed the studies reported herein to provide unambiguous evidence that the Galleria mellonella larva is a suitable model host for GAS.

Importantly, we documented a very high correlation of GAS strain virulence measured in wax worms and vertebrates (Fig. 3). Panels of well-characterized serotype M3 GAS strains isolated from humans and isogenic mutant GAS strains derived from a wild-type reference strain were tested. In nearly every case, the wax worm data were consistent with previously published results collected from mouse and monkey models. The one notable exception was carrier strain MGAS12502 (Fig. 3), which demonstrated unexpectedly high virulence in Galleria mellonella larvae. Whole-genome sequence analysis of this strain suggests that the probable genetic mechanism underlying its decreased virulence in mice, monkeys and humans is a 195 bp deletion that completely removes the amino-terminal region of the M-protein. As the major surface antigen of GAS, M-protein has been implicated in multiple virulence functions, including fibrinogen binding and complement inhibition. Since insects do not express these specific host factors, the M-protein mutation may be less detrimental to GAS virulence in wax worms than vertebrates. In contrast, the two other carrier strains tested have mutations affecting major transcriptional regulators which may inactivate critical virulence factor pathways such as tissue destruction that are likely important to mortality in all models (Fig. 3). Therefore, the carrier strain experiments illustrate an important example of the potentially imperfect recapitulation of GAS strain genotype-human disease phenotype relationships in Galleria mellonella larvae and emphasize the need to confirm all key results in higher animal models.

Tissue destruction has long been recognized as a characteristic feature of invasive GAS infection in humans, and disseminated disease is associated with poor prognosis. The histopathology studies performed on GAS-infected wax worms indicate that host tissue destruction can be readily investigated in this model. Compared to reference strain MGAS315, the less virulent GAS carrier and isogenic mutant strains caused considerably smaller lesions and less melanization (Fig. 4). This finding in the wax worm model is consistent with previous results using the carrier and isogenic mutant strains in vertebrate models (Flores AR, Musser JM, unpublished data). Of note, each of the genes targeted in the isogenic mutant strains either directly or indirectly inactivates a virulence pathway associated with tissue destruction.

An important trait of wax worms that directly bears on human microbial pathogenesis research is their tolerance for sustained incubation at 37°C. Smoot et al. have previously shown that the GAS transcriptome significantly changes when cultures are not grown at human body temperature. Growth at either 29°C or 40°C significantly altered the expression level of multiple proven and putative virulence factors, including genes implicated in carbohydrate and fatty acid metabolism, iron homeostasis and oxidative stress response, and cell wall and envelope biosynthesis. Similar temperature-dependent transcriptome changes have also been reported for Streptococcus agalactiae and Streptococcus pneumoniae, emphasizing the importance of this trait. In contrast, alternative invertebrate models host such as Drosophila melanogaster and Caenorhabditis elegans cannot be maintained at 37°C. Another beneficial feature of wax worms is the ease with which in vivo GAS gene expression studies can be performed. A growing body of evidence suggests an increased interest in performing GAS gene expression studies under human-relevant in vitro conditions or during in vivo infections. Infected hemolymph is easy to manipulate and enriched in GAS cells relative to host cells.
Materials and Methods

Bacterial strains. Serotype M3 GAS strains used in this study are described in Table 1. Wild-type reference strain MGAS315 (ATCC-BAA595) was recovered from a patient with streptococcal toxic shock syndrome; and its genome sequence has been reported. Generation of isogenic mutant strains and complemented mutant strains in the MGAS315 genetic background was previously reported, and they are also described in Table 1. To prepare GAS stocks for wax worm infection, strains were grown in Todd Hewitt broth supplemented with 0.2% yeast extract (THY, Becton, Dickinson and Company, Sparks, MD) at 37°C with 5% CO₂, to OD₆₀₀ = 0.5. GAS were collected by centrifugation, washed twice in phosphate buffered saline (PBS), suspended in 80% PBS/20% glycerol, aliquoted in cryovials, and stored at -80°C until use. The concentration of each stock strain (CFU/mL) was determined by serial dilution and colony counting of at least three aliquots. At the time of inoculation, stocks were diluted to the desired concentration and the infecting dose was confirmed by quantitative culture.

Galleria mellonella larva infection. Galleria mellonella larvae obtained from two vendors (Best Bet Inc., Blackduck, MN; and Knutson’s Recreational Sales Inc., Brooklyn, MI) were evaluated and found to perform equivalently. Larvae from a single vendor and lot were used for each experiment. After receipt, larvae were allowed to equilibrate for at least 24 h by storage in the dark at 10–12°C and used within 10 days. Only larvae measuring 2.0–2.5 cm in length and having a cream colored cuticle with minimal speckling or discoloration were used. Larvae were injected with 10 μL GAS through the left hindmost proleg into the hemocoel using a Hamilton 100 μL. 1710RN syringe fitted with a 29G needle. After inoculation, infected larvae were kept in vented 60 mm nematode petri dishes (Applied Scientific Products, San Francisco, CA) containing wood chips and incubated at 37°C and 0.5% CO₂ without humidification.

Galleria mellonella survival assays. For consistency, all survival assays were performed using larvae purchased from the same vendor (Best Bet Inc.). For survival and LD₅₀ assays, cohorts of 10 larvae were infected with each GAS strain-dose, and mortality was monitored every 12 h for 96 h. In the first survival assay, larvae were infected with serial ten-fold dilutions (10⁻⁷-10⁰ CFU in 10 μl PBS) of reference strain MGAS315. The 10⁻⁷-10⁴ CFU dose treatments were shown to provide discriminatory power in virulence, so only these four doses were used in all subsequent assays. For each survival experiment, each strain was tested in at least three independent assays, with reference strain MGAS315 used as a comparator. As a negative control, the first and last cohort of injected larvae in every assay was sham infected with sterile PBS. No more than one control larva died in any single replicate, and for simplicity, control groups are not shown in all figures. Survival curves were plotted using the Kaplan-Meier method and differences in survival were calculated using the logrank test (Prism 4.03, GraphPad Software Inc., La Jolla, CA). LD₅₀ was calculated using the Probit method (XLstat 2010, Addinsoft USA, New York, NY), and differences in strain virulence were compared using the Mann-Whitney test with p ≤ 0.05 considered to be statistically significant. The LD₅₀ of each GAS strain in wax worms was compared to its LD₅₀ in mice using Pearson’s correlation (XLstat).

Quantitative GAS culture. For the in vivo growth curve experiment, ~500 larvae were infected with 10⁷ CFU MGAS315 as described above. At each indicated time point, three pools of five surviving larvae each were randomly selected, transferred to a 5 ml Eppendorf tube containing 3 ml sterile PBS, homogenized, serially diluted and plated in duplicate on beta-select blood agar plates (Remel Products, Lenexa, KS). A subset of recovered colonies was confirmed as GAS using a latex agglutination kit (Becton, Dickinson and Company). Recovered CFUs/pool were plotted as mean ± standard error (Prism). For the CFU strain comparison experiment, larvae were infected with the indicated GAS strains, and four pools of five larvae each were harvested in the same manner. Differences in strain virulence were compared using the student’s t-test with p ≤ 0.05 considered to be statistically significant (XLstat).

In vivo gene expression. For in vivo GAS gene expression analysis, RNA was extracted from the hemolymph of infected larvae. Briefly, ~200 larvae were infected with 10⁷ CFU MGAS315 as described above. At 18 h post-inoculation, the hemolymph of ~100 surviving larvae was harvested by sterilizing the cuticle with isopropanol, puncturing the rear proleg with a 18G needle and collecting the hemolymph into a sterile 15 ml conical tube that contained 10 ml RNAprotect (Qiagen, Valencia, CA). After incubation on ice for 5 min, the hemolymph was centrifuged, and the GAS/hemocyte cell pellet was flash frozen. Approximately 3 ml hemolymph was collected. RNA was isolated as described previously using a FastPrep Blue Kit (MP Biochemicals LLP, Solon, OH) and purified using QiaShredder and RNeasy kits (Qiagen). The concentration and quality of RNA were assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA), and cDNA was created using Superscript III (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instruction. TaqMan quantitative real time PCR was performed as previously described in reference 13.
Histopathology of GAS-infected larvae. At the indicated time points, larvae were flash frozen and photographed using a Nikon inverted microscope (Nikon Inc., Melville, NY). For histopathology, larvae were fixed in formalin-aceto-alcohol for 24 hours, bisected and embedded in paraffin using standard techniques. Hematoxylin and eosin, Wright’s and Gram’s stained sections were examined by a pathologist blinded to specimen type using a BX5 microscope (Olympus Inc., Center Valley, PA). Representative fields were photographed with a DP70 camera (Olympus).

Acknowledgements
This work was supported in part by American Heart Association grant AHA0775045.

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