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Contribution of AmyA, an extracellular α -glucan degrading enzyme, to group A streptococcal host-pathogen interaction

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Abstract

α -glucans such as starch and glycogen are abundant in the human oropharynx, the main site of group A *Streptococcus* (GAS) infection. However, the role in pathogenesis of GAS extracellular α -glucan binding and degrading enzymes is unknown. The serotype M1 GAS genome encodes two extracellular proteins putatively involved in α -glucan binding and degradation; *pulA* encodes a cell-wall anchored pullulanase and *amyA* encodes a freely secreted putative cyclomaltodextrin α -glucanotransferase. Genetic inactivation of *amyA*, but not *pulA*, abolished GAS α -glucan degradation. The *amyA* strain had a slower rate of translocation across human pharyngeal epithelial cells. Consistent with this finding, the *amyA* strain was less virulent following mouse mucosal challenge. Recombinant AmyA degraded α -glucans into β -cyclomaltodextrins that reduced pharyngeal cell transepithelial resistance, providing a physiologic explanation for the observed transepithelial migration phenotype. Higher *amyA* transcript levels were present in serotype M1 GAS strains causing invasive infection compared to strains causing pharyngitis. GAS proliferation in a defined α -glucan-containing medium was dependent on the presence of human salivary α -amylase. These data delineate the molecular mechanisms by which α -glucan degradation contributes to GAS host-pathogen interaction including how GAS employs human salivary α -amylase for its own metabolic benefit.

Keywords

Streptococcus; salivary amylase; pharyngitis; translocation

Introduction

To successfully colonize or infect humans, bacteria must obtain nutrients from their host environment but understanding of the molecular mechanisms utilized by microbes to proliferate *in vivo* is limited (Xu *et al.*, 2003, Sonnenburg *et al.*, 2006, Shelburne *et al.*, 2008b). Given their presence at high concentrations in diverse human environments, complex carbohydrates may serve as key carbon sources for infecting microbes (Iyer & Camilli, 2007, Moyrand *et al.*, 2007, Shelburne *et al.*, 2006). Genome-wide investigations have demonstrated that genes encoding proteins involved in complex carbohydrate acquisition and utilization are highly expressed *in vivo* by diverse bacterial pathogens (Roos & Klemm, 2006, Rollenhagen & Bumann, 2006, Son *et al.*, 2007, Cho & Caparon, 2005). Further, for multiple human pathogens, genes encoding proteins involved in complex carbohydrate binding, transport, and metabolism contribute to microbial pathogenesis (Iyer & Camilli, 2007, Moyrand *et al.*, 2007, Munoz-Elias & McKinney, 2005). Therefore, investigation of microbial complex carbohydrate utilization pathways may generate novel insights into host-pathogen interaction.

Group A *Streptococcus* (GAS) causes infections in humans ranging from uncomplicated pharyngeal or skin infections to life-threatening bacteremia, pneumonia, and necrotizing fasciitis (Cunningham, 2000). The major site of GAS infection and colonization in humans is the oropharynx (Peter & Smith, 1977). α -glucans such as starch and glycogen are polysaccharides comprised of repeating D-glucose monomers linked by α -bonds and are present at high concentrations in the human oropharynx (Mormann & Muhlemann, 1981). As their molecular weight is typically > 100,000, to serve as an energy source α -glucans must be digested by extracellular enzymes to form smaller molecules that can be transported into the bacterial cell and enter into energy production pathways. Investigations in the 1950's determined that some GAS strains are capable of starch degradation, but the enzyme(s) responsible for GAS α -glucan degradation and subsequent transport are unknown (Crowley, 1950).

α -glucans may be degraded into glucoses linked in a linear fashion (i.e. maltodextrins) by enzymes termed amylases or pullulanases (Bertoldo & Antranikian, 2002) (Fig. S1). Alternatively, cyclomaltodextrin α -glucanotransferase (CGTase) enzymes degrade α -glucans into cyclic chains composed of glucose, i.e. cyclomaltodextrins (Qi & Zimmermann, 2005). The GAS serotype M1 strain MGAS5005 encodes at least two extracellular proteins putatively capable of α -glucan digestion (Ferretti *et al.*, 2001, Hytonen *et al.*, 2003, Lei *et al.*, 2000, Sumby *et al.*, 2005). Pula (M5005_SPy1682) is a cell-wall anchored pullulanase that in its purified form binds to and degrades glycogen and starch to linear maltodextrins (Hytonen *et al.*, 2003, van Bueren *et al.*, 2007), and AmyA (M5005_SPy1065) is a freely-secreted putative CGTase (Lei *et al.*, 2000). It is known that strain MGAS5005 transports and catabolizes linear maltodextrins up to 7 glucoses in length suggesting that a Pula initiated pathway for α -glucan catabolism could function in GAS (Shelburne *et al.*, 2007a). Alternatively, the genome of strain MGAS5005 also encodes proteins with similarity to enzymes in other bacterial species that are putatively involved in the transport of cyclomaltodextrins and their subsequent linearization to maltodextrins (Fiedler *et al.*, 1996). However, no physiological study of cyclomaltodextrin production and utilization in GAS or

any other Gram-positive pathogen has been performed. Thus, analysis of the GAS genome suggests that either AmyA or PulA or both could be critical to GAS α -glucan degradation.

Because of their predicted function and extracellular location, AmyA and PulA have been suggested to be GAS virulence factors (Ferretti et al., 2001, Hytonen et al., 2003, van Bueren et al., 2007, Hytonen *et al.*, 2006). Moreover the ability of GAS to degrade α -glucans has been postulated to contribute to GAS pathogenesis (Crowley, 1959, van Bueren et al., 2007). Therefore, we sought to define the molecular mechanisms by which GAS degrades α -glucans and to determine if α -glucan degradation contributes to GAS host-pathogen interaction. Our results demonstrate that AmyA-mediated α -glucan digestion influences GAS transepithelial migration and that GAS has adapted to use a host enzyme to initiate α -glucan catabolism.

Results

Analysis of GAS genome regions encoding genes putatively involved in extracellular α -glucan degradation

In the sequenced genome of the serotype M1 strain MGAS5005, there are two genes encoding putative extracellular α -glucan degrading enzymes, AmyA, a freely secreted, putative CGTase, and PulA, a cell-surface pullulanase (Sumby et al., 2005). *amyA* is one of 7 contiguous genes that encode proteins putatively involved in cyclomaltodextrin formation, transport, catabolism and gene regulation (Fig. 1). The *pulA* gene is about 575 kb away from *amyA* and is located next to genes putatively involved in linear maltodextrin degradation and transport. For the purposes of this manuscript, we will refer to the region comprising the open reading frames M5005_spy1061 to M5005_spy1067 as the cyclomaltodextrin (CMD) gene region and the region comprising M5005_spy1680 to M5005_spy1682 as the pullulanase gene region. The CMD gene region is immediately downstream of six contiguous open reading frames, M5005_spy1055 to M5005_spy1060, shown to be involved in linear maltodextrin transport and metabolism, which will be referred to herein as the linear maltodextrin (LMD) gene region (Shelburne et al., 2007a).

Only GAS strains encoding AmyA degrade starch

Previous investigators have associated the pathogenesis of GAS infection with starch degrading activity, but there is no information regarding the mechanism by which GAS breaks down starch or other α -glucans (Crowley, 1959). To begin to investigate the molecular basis of GAS α -glucan metabolism, we determined the starch degradation capacity for 72 GAS strains comprising 28 of the most common M serotypes isolated in a recent survey of GAS North American pharyngeal isolates (Shulman *et al.*, 2004). Strains from 10 of the 28 tested M serotypes, including the serotype M1 strain MGAS5005, degraded starch (Table S1) (Fig. 2). Next we used PCR to test for the presence of the *amyA* and *pulA* genes in all 72 strains. The *pulA* gene was amplified from all strains studied (data not shown). In contrast, *amyA* was only amplified from strains of the M serotypes that hydrolyzed starch (Table S1). Therefore, the presence of *amyA*, but not *pulA*, correlated perfectly with the ability of GAS to degrade starch.

AmyA is the major GAS extracellular α -glucan degrading enzyme and degrades α -glucans to cyclic maltodextrins

To assess whether AmyA is required for GAS α -glucan degradation, we created a *amyA* isogenic mutant strain (strain 5005.100, Table 1) from the parental serotype M1 strain MGAS5005 via non-polar insertional mutagenesis (Lukowski *et al.*, 2000). Additionally, we used a plasmid capable of replicating in GAS to create the genetically complemented strain 5005.100(pSS100) (Chaffin & Rubens, 1998). The doubling time during exponential growth and final growth densities of MGAS5005 in THY medium, glucose-medium, maltodextrin-medium, or in human saliva were not statistically different from strain 5005.100 (Table S2). Insertional mutagenesis of *amyA* abolished the ability of strain MGAS5005 to hydrolyze starch using starch/THY plates whereas providing *amyA* *in trans* to strain 5005.100 restored starch hydrolysis activity (Fig. 2). No significant difference in starch degradation was observed between parental strain MGAS5005 and its *pula* isogenic mutant derivative (strain 5005.200) (Fig. 2). We have previously shown that, in strain MGAS5005, *pula* transcript level is increased approximately 75-fold during growth in a maltodextrin-medium compared to a standard laboratory medium, which raises the possibility that the lack of observed Pula contribution to α -glucan degradation might have resulted from low Pula expression (Shelburne *et al.*, 2007b). However, we still could detect no difference in starch degradation between strain MGAS5005 and strain 5005.200 when starch/maltodextrin-medium plates were assayed demonstrating that increasing Pula expression did not reveal a role for Pula in GAS α -glucan digestion under the conditions tested (Fig. S2A).

We next sought to quantitate the contribution of AmyA to GAS α -glucan degradation and to determine whether the resultant digestion products were linear or cyclomaltodextrins. Similar to our findings using agar plates, the *amyA*-positive strains MGAS5005 and 5005.100(pSS100) degraded starch to a significantly greater extent than the *amyA*-negative strains 5005.100 and 5005.100(pDC123) when grown in either THY or a maltodextrin-medium (Fig. 2B, Fig. S2B). Incubation of strain MGAS5005 with starch or glycogen did not result in an appreciable increase in reducing (i.e. linear) sugar formation (data not shown). Rather, degradation of starch by strain MGAS5005 or 5005.100(pSS100) resulted in the production of β -cyclomaltodextrins (Fig. 2C, Fig. S2C). To definitively establish the role of AmyA in the production of β -cyclomaltodextrins, we overexpressed and purified mature GAS AmyA (Fig. 2D). Consistent with our strain data, purified AmyA degraded starch to β -cyclomaltodextrins (Fig. 2E, 2F). Next, purified AmyA and human salivary α -amylase, the major human α -glucan degrading enzyme in the oropharynx, were run in a non-denaturing gel and then allowed to diffuse overnight into a starch agar plate. Staining of the plate showed that starch degradation occurred only at the site of the band representing purified AmyA and human salivary α -amylase (Fig. 2G). Taken together, we conclude that under the tested conditions AmyA is the major GAS α -glucan degrading enzyme and that the digestion of α -glucans by AmyA results in the formation of cyclic maltodextrins.

Loss of genes in the cyclomaltodextrin region likely occurred because of transposon duplication resulting in two GAS lineages

Our finding that *amyA* is present only in a limited number of M serotypes raised the question of how such a genetic arrangement came to occur. To address this we used PCR

amplification spanning the CMD region to determine if all seven CMD genes are absent from the 37 strains of 16 M serotypes for which *amyA* amplification failed. The resultant amplicons indicated that the CMD region genes (i.e. M5005_spy1061 to M5005_spy1067) are absent en bloc from all 37 strains (Fig. 3, data not shown). The G+C content of the CMD region (9.3 kbp) is 38.08% whereas the G+C content of the core GAS genome is 38.59%. This difference is not statistically significant by either χ^2 ($P = 0.60$) or Student's *t*-test ($P = 0.11$) analyses. Moreover the dinucleotide composition (i.e. "genomic signature") of the CMD region does not differ significantly from the core genome (Table S3). These findings are consistent with the CMD genes being either the ancestral GAS condition or acquired from an organism with a GAS-like nucleotide composition. BLAST analysis found that among fully sequenced bacteria, the GAS CMD region genes are conserved in content and genome context only in a strain of the closely related group C *Streptococcus zooepidemicus* (Beres *et al.*, 2008).

To gain further insight into the molecular basis of CMD region presence or absence, we compared the sequence of gene regions flanking the CMD genes in CMD-negative and CMD-positive strains (Fig. 3A). The nucleotide position of discontinuity between the CMD positive and negative genomes is the same for both the 5' - (100 bps downstream of *malG*) and 3'-end breakpoints (413 bps upstream of M5005_spy1068). That is, the indel site is in the exact same nucleotide position in all fully sequenced GAS strains. This finding is consistent with a single ancestral genetic event as repeated acquisition at the exact same site in all strains would be extremely unlikely. Analysis of the residual genetic material between the cognates of M5005_spy1060 (*malG*) and M5005_spy1069 in the sequenced CMD-negative strains revealed that all CMD-negative strains have two transposase-like elements in this region. Cognates of the transposase M5005_spy1068 (shown in blue in Fig. 3A) are present in the same location in all GAS strains, regardless of CMD gene status and have a high degree of sequence identity (shown in Fig. 3A). In contrast, the transposase gene immediately downstream of *malG* is present at this loci only in CMD-negative strains. This transposase gene (shown in red in Fig. 3A) shares ~98% nucleotide identity over 955 bps with a transposase gene present near the origin of replication (M5005_spy1862, *parB/dnaA* gene region). The transposase near the origin of replication is present in all sequenced GAS strains. Thus, the CMD-negative strains have two copies of this transposase gene (one near the origin of replication and one in lieu of the CMD genes) whereas the CMD-positive strains only have the copy near the origin of replication. Therefore the genetic content of the CMD-negative strains suggests that the putative ancestral event leading to the loss of the CMD genes likely involved homologous recombination among transposases resulting in en bloc deletion of M5005_spy1061 to M5005_spy1067 leading to two branches of GAS (e.g. CMD-positive and CMD-negative strains). Of note, the gene encoding the fibronectin binding protein Fba is present in the same M protein serotype distribution as *amyA*, further supporting the concept of two distinct GAS lineages (Terao *et al.*, 2001).

***amyA* transcript levels are high during the stationary phase of growth, and *amyA* is expressed in humans with pharyngitis**

To begin to study the role of AmyA in host-pathogen interaction, we determined *amyA* transcript levels under a variety of conditions including from patients with GAS pharyngitis.

In contrast to previous investigations of the LMD region genes and *pulA*, we found negligible medium-dependent differences in *amyA* transcript levels for strain MGAS5005 (Fig. 4) (Shelburne et al., 2007b). Under all conditions tested, maximal *amyA* transcript levels were noted to be present during the stationary phase of growth (Fig. 4A). Similarly, high *amyA* transcript levels were found in patients with GAS pharyngitis caused by strains of M serotypes that contain the CMD genes (Fig. 4B). Thus, the high *amyA* transcript levels observed in patients with pharyngitis are likely due to constitutive expression in stationary phase rather than in vivo induction as has been observed for other carbohydrate utilization genes (Shelburne et al., 2008a).

Insertional inactivation of *amyA* does not affect the ability of GAS to colonize the mouse oropharynx but does affect the development of bacteremia following mucosal challenge

To this point, we had established that as the leading cause of pharyngitis, GAS is among a select few Gram-positive bacterium that encode AmyA and that *amyA* is expressed at high levels during GAS pharyngitis. Therefore, we next tested the hypothesis that AmyA contributes to GAS murine oropharyngeal colonization. To this end, we inoculated the nasopharynx of 35 adult outbred CD-1 mice each with either strain MGAS5005, 5005.100, or 5005.100(pSS100) and assayed for colonization by performing serial swabs of the oropharynx over a period of 14 days to ascertain the number of GAS present. Surprisingly, there was no statistically significant difference in either the proportion of mice testing positive for GAS colonization or in the average number of CFUs recovered per mouse between the three strains (Fig. 5A, data not shown). However, the overall mortality rate for mice infected with strains MGAS5005 and 5005.100(pSS100) was higher compared to strain 5005.100 (Fig. 5B). Analysis of the blood of dead or dying mice invariably revealed high numbers of GAS whereas healthy animals did not have GAS in their bloodstream. Taken together, these data do not support the hypothesis that AmyA contributes to GAS oropharyngeal colonization in the mouse. Rather, the data suggest that AmyA facilitates entry of GAS into the bloodstream.

***amyA* transcript levels are increased in invasive serotype M1 GAS strains, and AmyA contributes to epithelial translocation of strain MGAS5005**

Serotype M1 GAS strains can be broadly divided into pharyngeal and invasive isolates, with many invasive isolates having mutations in the control of virulence (CovR/S) two-component gene regulatory system resulting in derepression of genes encoding factors critical to the pathogenesis of invasive infection (Sumby et al., 2006). For example, strain MGAS5005 has a nucleotide insertion mutation in the *covS* gene that results in a truncated, non-functional CovS protein (Sumby et al., 2006). Given that *amyA* contributed to strain MGAS5005 bloodstream invasion, we next used real-time QRT-PCR to test the hypothesis that M1 strains with an “invasive” CovR/S system phenotype have higher transcript levels of *amyA* compared to strains with a “pharyngeal” phenotype (see Table 1 for strain description). For the mid-exponential, late-exponential, and stationary growth phases, we found that the “invasive” M1 strains had significantly higher levels of *amyA* transcript compared to the “pharyngeal” strains (Fig. 5C). These data are consistent with a previous investigation that found higher *amyA* transcript levels in a *covR* isogenic mutant strain compared to the parental strain MGAS5005 (Graham et al., 2002).

The presence of GAS in the bloodstream of the experimentally infected mice indicated that GAS had breached the airway epithelial cell barrier. Therefore, we next tested the hypothesis that strains MGAS5005, 5005.100, and 5005.100(pSS100), and 5005.100(pDC123) differ in epithelial translocation capacity. There was no significant difference in the ability of the four strains to cross a pharyngeal epithelial cell monolayer when the experiment was performed in a standard minimal medium (Fig. 6A). For each of the strains, the translocation of GAS across the pharyngeal cell layer was associated with an increase in epithelial cell permeability as determined by transepithelial electrical resistance (TEER) and fluorescein-dextran assays (Fig. 6B, 6C) which is consistent with a previous investigation of GAS epithelial cell translocation (Cywes & Wessels, 2001). When starch was included in the medium, strain MGAS5005 and 5005.100(pSS100) translocated earlier and at higher numbers compared to the strains lacking *amyA* (Fig. 6D). The earlier appearance of the *amyA*-positive strains was temporally related to a decrease in TEER and increase in fluorescein-dextran measurements indicating an accelerated loss of integrity of the epithelial cell monolayer (Fig. 6E, 6F). The addition of starch to the minimal medium did not increase GAS apical cell density for any of the strains compared to minimal medium alone (data not shown). Moreover, there were no significant CFU differences between the four strains in the apical compartment at any time point tested, indicating that increased bacterial burden did not account for the observed differences in cells in the basolateral compartment (data not shown). Therefore, we conclude that, in the presence of an α -glucan, AmyA augments the translocation capacity of an invasive M1 serotype GAS strain by a growth-independent mechanism.

The contribution to transepithelial invasion by AmyA is likely due to the production of cyclomaltodextrins

Cyclomaltodextrins interact with eukaryotic cell membranes leading to altered permeability and even toxicity (Matilainen *et al.*, 2008). We hypothesized that cyclomaltodextrin formation by the AmyA(+) strains contributed to their accelerated translocation rate compared to AmyA(-) strains. Indeed, the addition to the medium of β -cyclomaltodextrins at a concentration consistent with the β -cyclomaltodextrin production capacity of strain MGAS5005 restored the translocation rate of the AmyA(-) strains to wild-type levels (Fig. 6G). To determine if cyclomaltodextrins directly affected epithelial cell permeability, we incubated D562 pharyngeal epithelial cells with β -cyclomaltodextrins. The presence of β -cyclomaltodextrins, but not linear maltodextrins, led to increased permeability as assessed by accumulation of fluorescein-dextran in the basolateral compartment of the transwell and by a decrease in TEER (Fig. 6H). Therefore, we conclude that AmyA mediated α -glucan digestion results predominantly if not exclusively in the formation of cyclic rather than linear maltodextrins (see Fig. 2). Moreover, cyclomaltodextrins produced by AmyA α -glucan digestion increase pharyngeal epithelial cell barrier permeability resulting in enhanced GAS translocation capacity.

GAS utilizes human salivary α -amylase to initiate catabolism of α -glucans

During the translocation experiments, we noted that the addition of starch to minimal medium did not increase GAS cell density, suggesting that GAS was not catabolizing the cyclomaltodextrins produced by AmyA. We tested this hypothesis by growing strains

MGAS5005 and 5005.100 in a carbohydrate free chemically defined medium supplemented with various α -glucans. Strains MGAS5005 and 5005.100 were unable to grow in a medium in which soluble starch, glycogen, or pullulan was the sole carbon source (Fig. 7, data not shown). In contrast, the three α -glucan-media were capable of supporting bacterial growth as demonstrated by the growth of *Klebsiella oxytoca* strain M5a1, a bacterium that catabolizes starch via an actively secreted CGTase (Fig. 7) (Fiedler et al., 1996). We next tested the hypothesis that the lack of α -glucan catabolism was due to the inability of GAS to catabolize cyclomaltodextrins. Indeed, we found that strain MGAS5005 was unable to grow when cyclomaltodextrins (either α -, β -, γ -types) were the sole carbon source, with *K. oxytoca* again serving as the positive control (Fig. 7, data not shown) (Pajatsch *et al.*, 1998). To ensure that the results were not unique to strain MGAS5005, we tested strains from the 10 distinct *amyA*+ M serotypes for growth in starch- and cyclomaltodextrin-media. None of the strains was able to catabolize starch or cyclomaltodextrins (data not shown). These data demonstrate that whereas *amyA* containing GAS strains can degrade α -glucans via AmyA, under the conditions tested GAS is unable to catabolize the cyclomaltodextrins generated from α -glucan digestion.

The inability of GAS to catabolize α -glucans suggested that it might rely on a host enzyme to initiate α -glucan degradation. Human salivary α -amylase degrades α -glucans to linear maltodextrins and has the highest concentration of enzymes present in human saliva (approximately 200 U/mL) (Humphrey & Williamson, 2001). In light of the known ability of GAS to proliferate in human saliva, we hypothesized that GAS would grow in an α -glucan-medium supplemented with human salivary α -amylase (Shelburne *et al.*, 2005a). As hypothesized, the addition of physiologic doses of purified salivary α -amylase to either a starch- or glycogen-medium resulted in growth of strain MGAS5005 (Fig. 7). Neither the addition of α -glucan without α -amylase nor α -amylase without α -glucan resulted in detectable growth of strain MGAS5005 (data not shown). Thus, the data indicate that strain MGAS5005 can use α -glucans for growth when the host provides an enzyme that initiates degradation of α -glucans to linear maltodextrins, which have been shown previously to serve as a GAS carbon source (Kaczmarek & Rosenmund, 1977, Shelburne et al., 2007a).

Discussion

As numerous genome-wide analyses have suggested that complex carbohydrate utilization proteins contribute to infectivity, there has been significant investigation recently into the relationship between complex carbohydrate utilization and virulence in an array of human microbial pathogens (Iyer & Camilli, 2007, Moyrand et al., 2007, Munoz-Elias & McKinney, 2005). Genome-wide studies have identified streptococcal α -glucan degrading enzymes as putative virulence factors, but detailed analysis of the role of such enzymes in microbial pathogenesis have been lacking (Hava & Camilli, 2002). Therefore, we sought to understand the molecular mechanism of GAS extracellular α -glucan utilization and whether α -glucan degradation contributes to GAS host-pathogen interaction. Our data demonstrate that the freely secreted, α -glucan degrading CGTase AmyA influences GAS pathogenesis through a catabolism independent mechanism (Fig. 8).

One of our key discoveries was that AmyA is the primary GAS α -glucan degrading enzyme and that cyclomaltodextrins produced by AmyA α -glucan degradation disrupt human pharyngeal epithelial cell integrity resulting in enhanced bacterial translocation. The development of invasive disease following GAS pharyngitis is a rare, but potentially lethal complication (Carapetis *et al.*, 2005). It was recently discovered that, at least for serotype M1 and M3 strains, some GAS strains causing invasive disease have mutations in the CovR/S system which result in derepression of numerous virulence factors such as *amyA* (Sumby *et al.*, 2006, Miyoshi-Akiyama *et al.*, 2006). In addition to *amyA*, expression of genes encoding the hyaluronic acid capsule is increased in invasive GAS strains (Sumby *et al.*, 2006). A previous investigation identified the hyaluronic acid capsule as being a critical contributor to GAS translocation capacity (Cywes & Wessels, 2001). Similar to that study, we found that GAS epithelial cell translocation was associated with a loss of integrity of the epithelial cell layer suggesting that entry of GAS into deeper host tissues results from epithelial cell disruption rather than passage through intact host cells, such as occurs for *Streptococcus pneumoniae* (Beisswenger *et al.*, 2007). If alterations in the CovR/S system occur prior to epithelial translocation, then increased expression of the hyaluronic acid capsule along with increased cyclomaltodextrin production by AmyA may contribute to the subsequent development of invasive disease for AmyA-containing strains.

We determined that an entire genetic lineage of GAS strains lack the *amyA* gene and other CMD region genes and that gene loss was likely due to a duplication of a transposon located near the origin of replication resulting in en bloc deletion of the CMD region. The fact that all GAS strains sequenced to date that lack the CMD region genes differ from CMD-positive strains starting at the exact same site strongly suggests that a one-time genetic event occurred during GAS evolution leading to two separate GAS branches. Despite the fact that AmyA-positive GAS strains produce cyclomaltodextrins from starch, GAS appears unable to catabolize the cyclomaltodextrins, suggesting a loss of transport ability or subsequent degradative processes. Examination of the GAS genome (Fig. 1) reveals that all genes necessary for CMD transport and utilization seem to be present and intact. Indeed, we have recently found that some closely related group C and G streptococcal strains are able to catabolize starch and cyclomaltodextrins and have a similar CMD gene region to GAS (Shelburne, unpublished data). Thus, the reason for the inability of AmyA-encoding GAS strains to catabolize cyclomaltodextrins remains unclear and is an area of active investigation. The high level of salivary α -amylase in the human oropharynx means that GAS strains infecting or colonizing the oropharynx will be exposed to an α -glucan degrading enzyme thereby obviating the need for GAS to initiate α -glucan digestion. We have previously demonstrated that GAS had adapted its maltodextrin transport mechanism to optimally bind and transport linear maltodextrins resulting from α -glucan digestion by human salivary α -amylase (Fig. 8) (21). Therefore, the CMD genes may have been critical when GAS existed under different metabolic conditions, but GAS now appears to rely on its human host and the linear maltodextrin gene region for α -glucan catabolism, obviating the need for the cyclomaltodextrin pathway (Fig. S1). These findings suggest that the loss of AmyA and other CMD region genes may be part of the evolutionary process of GAS adaptation to its human host.

In summary, we have discovered the molecular basis for GAS α -glucan degradation and identified how GAS α -glucan degradation contributes to host-pathogen interaction. These data extend the growing understanding of the close links between nutrient catabolism and microbial pathogenesis in humans. Further investigation of these links may provide the basis for preventive or therapeutic strategies.

Experimental procedures

Bacterial strains and culture media

Bacterial strains and plasmid used in this study are listed in Table 1 and Table S1. The genomes of the invasive serotype M1 strain MGAS5005 and pharyngeal serotype M1 strain MGAS2221 have been sequenced (Sumby et al., 2005, Sumby et al., 2006). GAS M serotype was determined by sequencing of the *emm* gene. *Klebsiella oxytoca* strain M5a1 was obtained from ATCC (Fiedler et al., 1996). GAS was grown on Trypticase soy agar containing 5% sheep blood (BSA; Becton Dickinson) or in Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY; Difco) whereas *K. oxytoca* was grown in Luria-Bertani broth (LB; Difco). Spectinomycin or chloramphenicol (Sigma) antibiotic was added when appropriate to THY agar or broth at a concentration of 150 μ g/mL and 4 μ g/mL, respectively. Ultra-pure carbohydrates (Sigma) were added at a concentration of 0.5% (wt/vol) to a carbohydrate-free preparation of a commercially available chemically-defined medium (CDM; JR Biosciences) (Vise et al., 2003). For the purposes of this manuscript, we will use the terms glucose-medium, maltotriose-medium, starch-medium etc., to refer to the carbohydrate-free CDM supplemented with the specified carbohydrate. We have previously demonstrated that GAS cannot grow in the CDM without the addition of exogenous carbohydrates (Shelburne et al., 2007a). When indicated, purified human salivary α -amylase (Sigma) was added to CDM at physiologic concentration (200 U/mL) (Takai et al., 2004, Soderling et al., 1993).

Analysis of α -glucan degradation and cyclomaltodextrin production by GAS strains

GAS starch degrading activity on solid medium was assessed by plating 10 μ l of overnight THY culture onto THY or CDM-maltodextrin agar plates containing 0.5% soluble starch (Sigma). The plates were incubated overnight at 37°C, flooded with iodine, and examined for a zone of clearing (Glor et al., 1988). Starch and glycogen degradation by various GAS strains were assessed in a liquid medium consisting of THY or CDM supplemented with 0.5% wt/vol soluble starch via iodine staining with absorbance measured at 660 nm using α -glucan digestion by human salivary α -amylase as the standard (Thiemann et al., 2004). The production of linear maltodextrins from the digestion of starch or glycogen was assessed by measuring reducing sugar formation using the dinitrosalicylic acid assay with α -glucan digestion by human salivary α -amylase as the standard (Miller, 1959). β -cyclomaltodextrin spectrophotometric assays with purified cyclomaltodextrins as the standard were performed as described with 1 unit defined as the activity needed to produce 1 mg of β -cyclomaltodextrin from 1 mL of 0.5% wt/vol starch solution in 15 min (Makela et al., 1987). All α -glucan degradation experiments were done at least in triplicate on three separate occasions.

Creation of isogenic mutant and complemented mutant strains

Strain 5005.100 (*amyA* isogenic mutant) was created by non-polar insertional mutagenesis from strain MGAS5005 using the PCR splicing overlap extension method described by Kuwayama et al. with the spectinomycin resistance cassette being amplified from plasmid pSL60 (Kuwayama *et al.*, 2002, Lukomski et al., 2000). A similar strategy was used to create strain 5005.200 (*pulA* isogenic mutant). The isogenic mutant strains were analyzed by Southern hybridization and DNA sequencing to confirm that the proper genetic construct was obtained (Fig. S3). For complementation purposes, we used plasmid pDC123 which encodes the chloramphenicol acetyl transferase (*cat*) gene for antibiotic selection and the *phoZ* gene for blue-white screening via alkaline phosphatase activity (Chaffin & Rubens, 1998). The complete *amyA* gene along with ~ 250 bps upstream and ~15 bps downstream were amplified from strain MGAS5005 chromosomal DNA using primers that introduced *EcoR*I and *Bam*H1 cut sites at the 5' and 3' ends respectively. The resulting PCR product was digested with *EcoR*I and *Bam*H1 (Invitrogen) and directionally cloned into the *EcoR*I and *Bam*H1 sites of plasmid pDC123. The resulting plasmid, named pSS100, was used to transform competent *amyA* cells to create strain 5005.100(pSS100). pDC123 lacking the *amyA* gene was used to transform strain MGAS5005 and the strain 5005.100 as controls.

Overexpression and purification of AmyA and assessment of α -glucan degrading activity

PCR primers that introduced *Xho*I and *Eco*R1 restriction endonuclease sites were used to amplify the mature (i.e. lacking the secretion signal sequence) *amyA* gene from strain MGAS5005. The resultant PCR product was digested with *Xho*I and *Eco*R1 and ligated into the pBAD-hisA plasmid (Invitrogen) that had also been digested with the same enzymes. The ligation product, called pBAD-amyA, was cloned into Novablue (Novagen) and then BL21 (Novagen) competent *Escherichia coli* cells with selection via ampicillin resistance. pBAD-amyA was analyzed via sequencing to ensure that the correct construct had been obtained. To isolate purified protein, a Ni-NTA Fast Kit (Qiagen) was employed per the manufacturer's instructions with the AmyA protein being present in the soluble fraction. Size exclusion chromatography was used to complete purification (Fig. 2D). The amount of AmyA present was determined using a Bradford assay (Bio-Rad). The same procedure was performed using the empty vector pBAD-hisA to ensure that any observed activity was not due to carry over from the plasmid. α -glucan degrading activity was analyzed by incubating varying amounts of purified protein with 1 mL of a 1% starch solution for 1 hr at 37°C and then using iodine staining. Determination of α -glucan degradation products were done using the dinitrosalicylic acid method (for linear maltodextrins) and phenolphthaline method (for cyclic maltodextrins) as noted above (Miller, 1959, Makela et al., 1987). For solid-phase determination of purified AmyA activity, indicated amounts of purified protein were loaded into a 12% Tris-Glycine pH 10.4 non-denaturing gel and run for 4 h at 15 mA. The gel was then placed on top of a 1% of starch-agar plate, left for 24 h to allow for protein diffusion into the plate, and stained with iodine to assess for starch degradation.

Assessment of *amyA* and *pulA* distribution and allelic diversity

The presence of *amyA* (M5005_spy1065 or spy1302 in serotype M1 strain SF370) and *pulA* (M5005_spy1680 or spy1972 in strain SF370) in diverse GAS strains was determined by

PCR amplification. Chromosomal DNA was isolated using a DNeasy kit (Qiagen). PCR primers used for *amyA* and *pulA* amplification and sequencing were designed on the basis of the serotype M1 strain MGAS5005 genome (Sumbly et al., 2005). As a positive control, all strains were assessed for the presence of the *malE* gene, previously found to be present in all strains used in this study (Shelburne et al., 2006). The *amyA* nucleotide sequence was determined from data obtained from both DNA strands using an Applied Biosystems 3730XL DNA Analyzer. Sequences were assembled and edited using Sequencher v4.5, and the inferred amino acid sequences were aligned using ClustalW. All sequence polymorphisms identified relative to strain MGAS5005 were confirmed by a second independent iteration of the entire DNA sequence determination process. Strains for which *amyA* failed to amplify using the initial primer pair were re-tested with an alternate *amyA* primer pair. Strains for which *amyA* failed to amplify using both the initial and alternate primers pairs, were further examined using a third pair of primers located in sequence flanking the cyclomaltodextrin genes that is conserved among the genomes of 12 sequenced GAS strains. Resultant flanking primer amplicons were examined for having a size (c.a. 1 kb) consistent with the lack of the cyclomaltodextrin utilization genes such as is the case for the M3 strain MGAS315 genome (Beres et al., 2002).

Assessment of gene similarities, codon usage, and nucleotide composition

Sequence alignments for assessing allelic diversity and identification of single nucleotide polymorphisms (SNPs) were performed using SeqScape v2.5 (Applied Biosystems) and MUMmer (Kurtz et al., 2004). Local best fit alignments of the α -glucan utilization genes studied with the sequenced GAS strains and the NCBI non-redundant sequence database were performed using BLAST (Altschul et al., 1990). Determination of nucleotide composition (mono- and di- nucleotide frequencies), and codon usage was determined using MacVector and CodonW (Rastogi, 2000). Global pairwise and multi sequence alignments were performed using ClustalW (Chenna et al., 2003).

RNA isolation and transcript level analysis

For transcript level analysis under diverse growth conditions, various GAS strains were grown as indicated. RNA was isolated and purified using an RNeasy kit (Qiagen) (Shelburne et al., 2005b). The quality and the concentration of RNA were assessed with an Agilent 2100 Bioanalyzer and by analysis of the A_{260}/A_{280} ratio. cDNA was reverse transcribed from RNA using Superscript III (Invitrogen) following the manufacturer's instructions. TaqMan quantitative real-time PCR (QRT-PCR) was performed with an ABI Thermocycler 7700 (Applied Biosystems) using the C_T method of analysis with the *proS* gene used as the internal control (Chaussee et al., 2001). To determine *amyA* transcript levels *in vivo*, GAS RNA was isolated from throat swabs obtained from six patients with GAS pharyngitis and analyzed as previously described (Virtaneva et al., 2003). The throat swabs were obtained from patients who gave their informed consent prior to participation in the study according to a protocol for human subjects approved by the Baylor College of Medicine Institutional Review Board. To ensure accuracy, all QRT-PCR primers and probes were tested to confirm similar amplification efficiencies using GAS genomic DNA as template. Primers and probes used are listed in Table S4. All transcript level analysis experiments were performed in quadruplicate on three separate occasions.

Growth of GAS strains in various media

All growth experiments were done in triplicate on three separate occasions for a total of nine replicates. For studies comparing GAS growth in THY or CDM, starter cultures were grown overnight in THY. Overnight cultures were inoculated into fresh medium to achieve a uniform starting OD₆₀₀ of 0.01. Growth was monitored by measuring optical density at 600 nm. GAS was grown in human saliva as previously described (Shelburne et al., 2005a). Healthy volunteers who gave their informed consent prior to participation in the study donated saliva according to a protocol for human subjects approved by the Baylor College of Medicine Institutional Review Board. Saliva pooled from at least four donors was used to minimize the effects of donor variation on study results.

Mouse pharyngeal mucosal challenge

All experiments in mice were performed according to a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee and were conducted with adult (18- to 20-g) female outbred CD-1 Swiss mice (Harlan Sprague-Dawley Inc.) (Shelburne et al., 2006, Lukowski et al., 2000). GAS strains were grown in THY and harvested at an OD₆₀₀ of approximately 0.5. The cells were washed once with sterile phosphate buffered saline (PBS) and suspended in PBS at 1×10^8 colony forming units (CFU)/ml. Both nares of each mouse were inoculated with 50 μ L of the GAS suspension (total inoculation 100 μ L, 1×10^7 CFU) with 35 mice in each group. The throat of each mouse was swabbed before inoculation and then at specific time periods thereafter. The throat swabs were streaked onto BSA, grown overnight, and β -hemolytic colonies were enumerated. For each plate, five of the resultant β -hemolytic colonies were then tested for the presence of GAS carbohydrate antigen via latex agglutination with all colonies testing positive indicating that all of the β -hemolytic colonies were GAS (BD Biosciences).

Transepithelial invasion assays

D562 cells (human oropharyngeal cell line, ATCC) were grown to confluence on 24 mm collagen Transwell inserts with 3.0 μ m pores in 6 well cell culture plates (Corning Costar) in Eagle's minimal essential medium (ATCC) with 10% fetal bovine serum (Lonza) supplemented with penicillin and streptomycin (100 IU/mL and 100 μ g/mL respectively, Mediatech). The integrity of the D562 monolayer epithelial barrier was determined by measuring the transepithelial resistance (TEER) using the Millicell-Electrical Resistance System (Millipore). The TEER value for the confluent D562 monolayer was $\sim 170 \Omega \text{ cm}^2$. Monitoring of cell barrier integrity was also performed using FITC-dextran (Sigma) which was added to the apical chamber at 1 μ M final concentration with measurement done in the basolateral chamber by removing 100 μ L and scanning at 521 nm in a Synergy HT microplate reader (Biotek). An intact barrier was generally achieved 14 days after inoculation of the cells. GAS translocation across the epithelial cell barrier was determined by growing GAS to mid-logarithmic growth phase, washing with PBS, and adding GAS resuspended in antibiotic free minimal medium to the apical chamber at a multiplicity of infection of 1 CFU per epithelial cell. The lower chamber was monitored hourly for the presence of GAS by removal of 100 μ L of media followed by direct plating onto BSA with the lower limit of detection being 10 CFU/ml. Starch was added to the apical chamber at a

0.5% wt/vol when indicated. 5 mM β -cyclomaltodextrin (Sigma) was added at indicated concentrations where noted. The choice of 5 mM β -cyclomaltodextrin was based on the capacity of strain MGAS5005 to generate β -cyclomaltodextrins from α -glucans (i.e. presumed physiologic concentration). The integrity of the epithelial cell barrier was followed using TEER and FITC measurements. All experiments were performed in six replicates for each strain on four separate occasions.

Statistical analysis

Growth, amylase activity, and RNA transcript levels were compared using Student's 2-sided *t*-test. For the murine mucosal challenge model, a repeated measures analysis was used to test for differences in the percent of animals colonized and in the number of CFU recovered per animal. Difference in the transepithelial invasion of various strains was determined using a repeated measured analysis. Statistical significance was assigned a 2-sided *P* value of 0.05 using Bonferroni's adjustment for multiple comparisons when appropriate. Statistical calculations were performed using NCSS software version 2004.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Schematic of the linear maltodextrin, cyclomaltodextrin and pullulanase gene regions in GAS serotype M1 strain MGAS5005 (Sumbly et al., 2005). *amyA* and *pulA* genes are indicated in white. M5005_spy numbers refer to open reading frame in the serotype M1 strain MGAS5005. ABC = ATP-binding cassette.

Fig. 2.

AmyA α-glucan digestion results in β-cyclomalto-dextrin formation. (A) GAS strains of indicated *amyA* and *pulA* genotype were grown overnight on THY agar plates supplemented with 0.5% starch. Iodine was added to plates and the presence of clearing was assessed for evidence of starch hydrolysis. (B) Colorimetric analysis of iodine staining following growth in THY supplemented with 1% starch was used to assess starch degrading activity in indicated GAS strains as described in *Experimental procedures*. (C) β-cyclomalto-dextrin production from starch by various strains was assessed using colorimetric analysis as

described in *Experimental procedures*. (D) GAS AmyA was cloned and overexpressed in *E. coli* and then purified to apparent homogeneity as described in *Experimental procedures*. Proteins were separated on a 12% polyacrylamide gel. (E) Degradation of starch by purified AmyA with empty pBAD-hisA vector as negative control. (F) Production of B-cyclomaltodextrin by purified AmyA with empty pBAD-hisA vector as negative control. (G) Purified AmyA was run under non-denaturing conditions as described in *Experimental procedures* and subsequently allowed to diffuse into a 1% starch agar plate. Iodine staining confirmed that starch degradation (bright areas) was limited to the gel locations corresponding to AmyA. Lanes are as follows; 1 = 5 μ l purified AmyA, 2 = 5 μ l human salivary α -amylase, 3 = 5 μ l empty vector. All starch degrading experiments were performed in triplicate on four separate occasions with data graphed being mean and error bars representing standard deviation.

Fig. 3.

Schematic of cyclomaltoextrin (CMD) region genes in various GAS strains. (A) Overview of CMD region in sequenced GAS strains. CMD(+) sequenced strains include serotype M1, M2, M4, and M28 strains (Ferretti et al., 2001, Sumby et al., 2005, Beres *et al.*, 2006, Green *et al.*, 2005). CMD(-) sequenced strains include serotype M3, M5, M6, M12, M18, and M49 strains (Beres et al., 2002, McShan *et al.*, 2008, Nakagawa *et al.*, 2003, Holden *et al.*, 2007, Banks *et al.*, 2004, Smoot *et al.*, 2002, Beres et al., 2006). CMD(+) and CMD(-) strains share CMD flanking region genes and a transposase gene (M5005_spy1068) shown in blue. All GAS strains sequenced to date have a second transposase gene (M5005_spy1862, shown in red) found near the origin of replication that is duplicated in place of the CMD region genes in CMD (-) strains. (B) PCR reactions to determine presence or absence of CMD genes. Lane designations: Serotype M1 strain MGAS5005 with *malX* primers (1), with CMD region flanking primers (2), with *amyA* primers (3); serotype M3 strain MGAS315 with *malX* primers (4), with CMD region flanking primers (5), with *amyA* primers (6). Small black arrows showing primer locations appear in (A).

Fig. 4.

Analysis of *amyA* transcript levels. (A) Serotype M1 strain MGAS5005 was grown to early-exponential (exp), mid-exponential, late-exponential, and stationary growth phase in indicated media in quadruplicate biologic replicates on three separate occasions (total of 12 samples at each time point). RNA was isolated, converted to cDNA, and transcript levels for *amyA* and the endogenous control gene *proS* were determined using TaqMan real-time PCR. Data graphed are mean $\pm$ standard deviation. (B) *amyA* transcript levels for 6 patients with GAS pharyngitis were determined by TaqMan real-time PCR. The M serotype of the

infecting GAS strain is shown in the circle. Data graphed are mean $\pm$ standard deviation with each experiment done with quadruplicate technical replicates on three separate occasions (total of 12 data points per sample).

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Fig. 5.

AmyA does not contribute to GAS colonization of the mouse oropharynx but does increase murine mortality following mucosal challenge. Adult outbred CD-1 mice ($n = 35$ per group) were intranasally inoculated with $\sim 1.0 \times 10^7$ CFU with indicated GAS strains. Mice oropharynxes were swabbed daily and plated onto BSA. Plates were incubated for 24 hr, and β -hemolytic colonies were counted and tested for GAS carbohydrate antigen using latex agglutination. (A) Percentage of mice with GAS isolated by day. P value refers to repeated measures analysis. (B) Percent mice surviving graphed by data with P value referring to χ^2 test done on day 14 of experiment. (C) Analysis of *amyA* transcript levels during growth in

THY at indicated growth phase in invasive vs. pharyngeal GAS strains (for strain description see Table 1). * = $P < 0.05$ at the mid-logarithmic, late-logarithmic, and stationary growth phases when comparing invasive vs. pharyngeal strains. Transcript level analysis was done using quadruplicate biologic replicates on three separate occasions with data graphed being mean $\pm$ standard deviation.

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Fig. 6.

AmyA increases GAS trans epithelial invasion in the presence of starch. GAS translocation across a D562 epithelial cell monolayer were performed as described in *Experimental procedures* using indicated strains. For (A), (B), and (C) experiments were performed in standard minimal medium. (A) Number of translocated GAS by indicated strain. (B) and (C) are indicators of epithelial integrity using transepithelial resistance (TEER) and fluorescent-dextran. For (D), (E), and (F) experiments were performed in minimal medium with 0.5% starch added. (G) 5 mM β -cyclodextrins were added to the starch-medium. (H) TEER and fluorescent measurements in the presence indicated concentrations of β -cyclodextrin without GAS. Transepithelial invasion assays were performed in six replicates per strain on four separate occasions. Data graphed are mean with error bars representing standard deviation. Asterisks indicate a P value of < 0.05 using a repeated measures analysis.

Fig. 7.

GAS uses human salivary α -amylase to initiate α -glucan catabolism. Data graphed are growth curves of GAS in various carbohydrate supplemented chemically defined media. Serotype M1 strain MGAS5005 was grown overnight in THY and then added at a 1:100 dilution into designated medium with growth monitored via OD₆₀₀ readings. Human salivary α -amylase was added at physiologic concentrations when indicated. β -CMD = cyclomaltoextrins. *Klebsiella oxytoca*, which is capable of growth in starch, is included as a positive control. All growth experiments were performed in triplicate on four separate occasions with mean $\pm$ standard deviation graphed.

Fig. 8.

Schematic of hypothesized role for α -glucan degrading enzymes in the pathogenesis of GAS pharyngitis. Degradation of α -glucans (such as starch or glycogen) by human salivary α -amylase results in the production of linear maltodextrins which can be used as an energy source for GAS proliferation. Alternatively, GAS AmyA α -glucan digestions leads to cyclic maltodextrin formation resulting in a decrease in epithelial transepithelial resistance and subsequent GAS translocation.

Table 1

Strains and plasmids.

Strain or plasmid	Description	Genotype	Reference
Strains			
MGAS5005	Invasive isolate, serotype M1	<i>covS</i> nt80	(Sumby et al., 2005)
MGAS5005(pDC123)	MGAS5005 with empty vector	MGAS5005, Cm ^r	(Shelburne et al., 2006)
5005.100	<i>amyA</i> strain	MGAS5005 <i>amyA</i> :: <i>spc</i>	This study
5005.100(pDC123)	<i>amyA</i> strain with empty vector	<i>amyA</i> , Spc ^r Cm ^r	This study
5005.100(pSS100)	<i>amyA</i> strain with complementation vector	<i>amyA/amyA</i> +, Spc ^r Cm ^r	This study
5005.200	<i>pulA</i> strain	MGAS5005 <i>pulA</i> :: <i>spc</i>	This study
MGAS294	Invasive isolate, serotype M1	V286F in <i>covS</i>	(Sumby et al., 2006)
MGAS315	Invasive isolates, serotype M3	Wild-type	(Beres et al., 2002)
MGAS2217	Invasive isolate, serotype M1	1 bp insert in <i>covS</i>	(Sumby et al., 2006)
MGAS2221	Pharyngeal isolate, serotype M1	Wild-type	(Sumby et al., 2006)
MGAS5322	Pharyngeal isolate, serotype M1	Wild-type	(Sumby et al., 2006)
MGAS5392	Pharyngeal isolated, serotype M1	Wild-type	(Sumby et al., 2006)
MGAS6184	Invasive isolate, serotype M1	A397V in <i>covS</i>	(Sumby et al., 2006)
MGAS9506	Pharyngeal isolate, serotype M	Wild-type	(Sumby et al., 2006)
<i>Klebsiella oxytoca</i> M5a1		Wild-type	(Fiedler et al., 1996)
Plasmids			
pSL60-1	Vector containing <i>aad9</i> gene	Spc ^r	(Lukomski et al., 2000)
pDC123	Chloramphenicol resistance vector	Cm ^r	(Chaffin & Rubens, 1998)
pSS100	<i>amyA</i> complementation vector	Cm ^r	This study
pBAD_amyA	pBAD vector with mature <i>amyA</i> sequence	Amp ^r	This study