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ORIGINAL ARTICLE

Oxygen-dependent phenotypic variation in group A streptococcus

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Background: The phenotypic heterogeneity of the human pathogen *Streptococcus pyogenes* [group A streptococcus (GAS)] is associated with bacterial virulence variation. During invasive GAS infection, mutations in the two-component regulatory system *covR/covS* leads to increases in hyaluronic acid capsule production, virulence genes expression, and lethality in the mouse infection model. Phenotypic variation of GAS is also found under *in vitro* culture conditions. However, whether a specific environmental factor is important for phenotypic variation is still unknown.
Methods: GAS968 is an *emm12*-type clinical isolate that converts from mucoid to hypermucoid morphology under *in vitro* culture conditions. To clarify whether morphology variation can be triggered by specific environmental signals, or whether different morphology variants would be selected under specific environmental stresses, GAS968 was cultured under different conditions, and the changes in the number of mucoid and hypermucoid colonies in the total bacterial population were analyzed.
Results: The ratio of mucoid and hypermucoid colonies of GAS968 in the total bacterial population changes dramatically under aerobic and anaerobic conditions. The decrease in the number of hypermucoid colonies in the total bacterial population under aerobic conditions is not caused by growth repression, suggesting that the morphology conversion of GAS968 is inhibited by oxygen.

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Conclusion: Phenotypic heterogeneity has been shown to contribute to invasive GAS infection. Our results suggest that oxygen-dependent morphology variation in GAS968 may have important roles in bacterial pathogenesis.

Introduction

Streptococcus pyogenes [group A streptococcus (GAS)] is an important human pathogen, causing diseases such as pharyngitis, tonsillitis, scarlet fever, cellulitis, necrotizing fasciitis, and toxic shock syndrome.[1,2] Overproduction of hyaluronic acid capsule in GAS changes the bacterial colony morphology from glossy to mucoid or matte form.[3,4] Hyaluronic acid capsule is an important virulence factor that allows GAS to escape from immune clearance. Therefore, mucoid or matte form GAS has been considered more virulent than GAS with glossy morphology.[5]

The expression of hyaluronic acid capsule is negatively regulated by the two-component regulatory system, CovR/CovS (CovR/S).[4] Mutation in the *covR/S* gene derepresses the expression of hyaluronic acid capsule but also upregulates the expression of many virulence factors such as streptolysin O (SLO) and DNase Sda1, resulting in increased resistance to immune clearance, virulence, and lethality in the mouse infection model.[6–9] Clinical studies showed that strains with mutations in *covR/S* genes were isolated more frequently from severe invasive streptococcal infections,[10–12] indicating that GAS strains with mucoid morphology are potentially more virulent and invasive. A recent study further showed that immune pressure, especially neutrophil, is critical to the selection of GAS that acquired *covR/S* mutations during infection.[13]

Phenotypic variation of GAS is also found under *in vitro* culture conditions. Cleary et al[14] showed that M1 GAS 90-131 strain segregates morphology distinguishable colonies during *in vitro* incubation. The larger colonies have increased capsule expression and have better internalization activity for human alveolar A549 epithelial cells when compared to small colonies.[14] In addition, their study showed that morphology conversion (from small to large colonies) cannot be controlled. Our previous study showed that the *emm* type 12 clinical isolate GAS968 also has the morphology variation property that converts morphology from mucoid to hypermucoid form under *in vitro* culture conditions.[15] In addition, the hypermucoid variant is more invasive than the mucoid variant in the mouse infection model.[15]

In the present study, we found that the morphology variation of GAS968 can be manipulated by changing the oxygen concentration in the culture medium. In addition, our results further showed that the decrease in the number of hypermucoid colonies in the total bacterial population is not caused by bacterial growth repression under aerobic conditions. The phenotypic variation has been shown to contribute to invasive GAS infections[6,14]; our results suggest that the oxygen-dependent morphology variation in GAS968 may have important roles in bacterial pathogenesis.

Methods

Bacterial strains and culture conditions

GAS strain 968is an *emm12*-type strain that has the G35D mutation in CovR.[15] GAS were cultured on trypticase soy agar with 5% sheep blood, in tryptic soy broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 0.5% yeast extract (TSBY), or in C medium[16] supplemented with/without 100 mg/dL glucose. Bacteria cultured under conditions of enhanced aeration in 250-mL flasks containing 10 mL broth and subjected to orbital shaking (200 rpm) in the ambient air at 37°C is defined as the aerobic culture condition.[17,18] Anaerobic culture condition indicates bacteria grown in the anaerobic jam with anaerobic atmosphere generation bags (AnaeroPack-Anaero; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 37°C.

RNA manipulations

RNA extraction from GAS and reverse transcription were performed using a previously described method.[19] Real-time polymerase chain reactions were performed in a 20-μL mixture containing 1 μL cDNA, 0.8 μL primers (10 μM), and 10 μL SensiFAST SYBR Lo-ROX premixture (Bioline Ltd., London, UK) according to the manual. The expression level of each target gene was normalized to *gyrA* and analyzed using the $\Delta\Delta C_t$ method (7500 software v2.0.5; Applied Biosystem, Thermo Fisher Scientific Inc., Foster City, CA, USA). Biological replicate experiments were performed from at least three independent RNA preparations in duplicate. The primers used for real-time polymerase chain reaction (*lctO*-F-1: 5′-ttgctgacaaagatggttcg-3′; *lctO*-R-1: ttctggcaggtcagttgttg; *gyrA*-F-3: 5′-cgtcgtttgactggtttgg-3′; *gyrA*-R-3: 5′-ggcgtgggttagcgtattta-3′) analysis were designed by Primer3 (v.0.4.0, http://frodo.wi.mit.edu) according to the MGAS5005 sequence (NCBI reference sequence: NC_007297.1).

Measurement of hydrogen peroxide

Hydrogen peroxide in bacterial culture supernatant was measured as previously described.[18] Supernatants from overnight-cultured bacteria were collected and filtered through 0.22-μm filters (Millipore, Billerica, MA, USA). The bacterial culture supernatant (180 μL) was mixed with 20 μL of 1× phosphate buffer solution containing 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (A-1888; Sigma-Aldrich, St. Louis, MO, USA) at 3 mg/mL and horseradish peroxidase (P-8250; Sigma-Aldrich) at 0.2 mg/mL, and incubated at room temperature for 20 minutes. The

hydrogen peroxide concentration was measured with the spectrophotometric method (A_{560}).

Measurement of glucose and lactate in culture supernatants

Bacteria were cultured in 10 mL of C medium with orbital shaking (200 rpm) at 37°C for 12–16 hours. After the incubation, 400 μL of the bacterial suspension was transferred to 10 mL of C medium (supplemented with 100 mg/dL glucose) and cultured under aerobic or anaerobic conditions. Bacterial supernatants were collected (3500 rpm for 10 min at 4°C) and filtered through a 0.22-μm filter (Millipore). Glucose and lactate concentration of bacterial supernatants were determined using the Rodox Glucose kit (Randox Laboratory Limited, London, UK) and Lactate colorimetric/Fluorometric assay kit (BioVision Inc., Milpitas, CA, USA), respectively, according to the manuals.

Statistical analysis

Statistical analysis was performed using the Prism software, version 4 (GraphPad, San Diego, CA, USA). A p value <0.05 (Student t test) was considered significant.

Results

Morphology variation under aerobic and anaerobic conditions

Our previous study found that the *emm12*-type GAS968 has a morphology variation property that spontaneously converts colony morphology from mucoid to hypermucoid under *in vitro* culture conditions.[15] In addition, 88–95% of bacteria recovered from the mucoid form GAS968 ($GAS968_{MU}$) infected mice showed a hypermucoid morphology, suggesting that this morphology variation contributes to the increase in bacterial virulence during infection.[15] To clarify whether the morphology variation of GAS968 could be triggered by specific environmental signals, or whether different morphology variants would be selected under specific environmental stresses, $GAS968_{MU}$ was cultured under different conditions and the changes in the number of mucoid and hypermucoid colonies in the total bacterial population were analyzed. Similar to our previous study, after $GAS968_{MU}$ was cultured in TSBY broth for 16 hours, about 20% of the colonies converted to the hypermucoid morphology (Fig. 1). We found that oxygen is an important environmental factor that affects the morphology variation property of $GAS968_{MU}$. Under aerobic conditions, less than 8% of bacterial colonies showed a hypermucoid morphology. When $GAS968_{MU}$ was cultured under anaerobic conditions, more than 70% of colonies converted to a hypermucoid morphology (Fig. 1).

Lactate oxidase expression and hydrogen peroxide production of mucoid and hypermucoid variants

We have previously demonstrated that the hypermucoid variant ($GAS968_{MA}$) is more sensitive to oxidative stress than $GAS968_{MU}$.[15] Under *in vitro* culture conditions, hydrogen peroxide produced by the bacterium itself is one of the major sources of oxidative stress. Previous studies have shown that some of the GAS isolates are capable of producing H_2O_2 via oxidized lactate by lactate oxidase under aerobic, glucose-limited conditions.[17,20,21] To clarify whether hydrogen peroxide is an environmental factor in selection of mucoid variants under aerobic conditions, the expression of the lactate oxidase gene (*lctO*) and the concentration of hydrogen peroxide in the culture supernatant were analyzed. Bacteria were cultured in C medium with agitation for 5 hours (exponential phase of growth), and the *lctO* expression was analyzed with quantitative reverse-

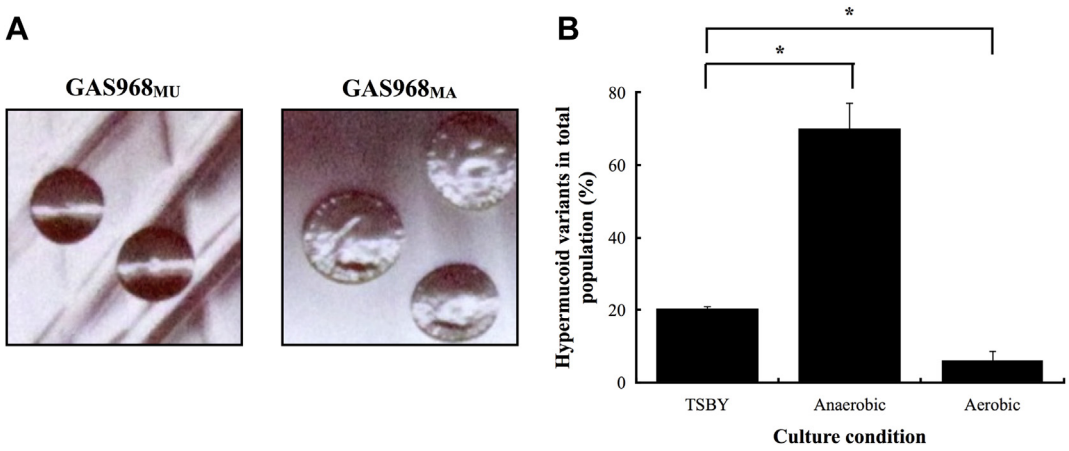

Figure 1. Colony morphology of mucoid ($GAS968_{MU}$) and hypermucoid ($GAS968_{MA}$) variants, and the ratio of hypermucoid variants in different culture conditions. GAS968 was passaged on blood agar plates and incubated at 37°C with 5% CO_2 supplementation for 12–16 hours. (A) Colonies with hypermucoid and mucoid morphology. (B) Ratio of mucoid and hypermucoid colonies in total bacterial population under normal (TSBY broth, 5% CO_2 without agitation), anaerobic (anaerobic jar, without agitation), and aerobic (ambient air, with vigorous shaking) culture conditions. Mucoid variant $GAS968_{MU}$ were cultured in TSBY or C medium (aerobic or anaerobic conditions) for 12–16 hours, and the number of hypermucoid colonies was calculated with the plating method.*$p < 0.05$.

transcription polymerase chain reaction. The result showed that GAS968$_{MU}$ had a higher *lctO* expression compared with GAS968$_{MA}$ (Fig. 2A). Expression of *lctO* in GAS has been shown to be repressed in the presence of glucose.[17] In agreement with previous studies, our results also showed that the expression of *lctO* was repressed in both GAS968$_{MU}$ and GAS968$_{MA}$ in the C medium supplemented with 100 mg/dL glucose (Fig. 2B and C). The increase in *lctO* expression in GAS968$_{MU}$ may relate to the increase in hydrogen peroxide production. To clarify whether GAS968$_{MU}$ may produce more H_2O_2 compared with GAS968$_{MA}$, the concentration of hydrogen peroxide in bacterial culture supernatants was analyzed further. However, results showed that there was no detectable H_2O_2 in the culture supernatants collected from both GAS968$_{MU}$ and GAS968$_{MA}$ under aerobic or anaerobic culture conditions (Table 1).

Table 1 Hydrogen peroxidase concentration in bacterial culture supernatants after 16 hours of incubation.

Strain/culture condition	Aerobic condition (A_{560})	Anaerobic condition ($_{A560}$)
GAS968$_{MU}$	0.015 ± 0.005	0.013 ± 0.0001
GAS968$_{MA}$	0.015 ± 0.003	0.010 ± 0.003

Growth of GAS968$_{MU}$ and GAS968$_{MA}$ under aerobic and anaerobic culture conditions

GAS968$_{MU}$ and GAS968$_{MA}$ are hydrogen peroxide non-producers (Table 1). Therefore, hydrogen peroxide is not an important factor to repress the growth of hypermucoid variants under aerobic conditions. To clarify whether the decreased fitness under aerobic conditions was the major mechanism for selection of hypermucoid variants, the bacterial growth under aerobic and anaerobic conditions were analyzed. After 7 hours of incubation, the number of GAS968$_{MU}$ colony is 2.19 ± 0.51-fold and 6.09 ± 1.12-fold higher than that of GAS968$_{MA}$ under aerobic and anaerobic condition, respectively. Although the hypermucoid variant is the dominant population under the anaerobic condition, the bacterial growth rates in the log to exponential phase under anaerobic conditions of both GAS968$_{MU}$ and GAS968$_{MA}$ were 1.41- to 1.64-fold faster (8.04 ± 1.57 and 7.89 ± 1.16 minutes, respectively) relative to those cultured under the aerobic condition (13.20 ± 4.01 and 11.15 ± 1.81 minutes, respectively). In addition, in the exponential to stationary phase, GAS968$_{MU}$ still had a better growth activity than that of GAS968$_{MA}$ under both aerobic and anaerobic conditions (data not shown). These results indicate that population change of the hypermucoid variant in the total bacterial population may not be caused by the decrease of its fitness, and support the notion that hydrogen peroxide may not be a critical factor to select the mucoid variant under aerobic conditions.

Glucose utilization and lactate accumulation of GAS968$_{MU}$ and GAS968$_{MA}$

GAS is a member of lactic bacteria that majorly ferment glucose and produce lactate during incubation.[22]

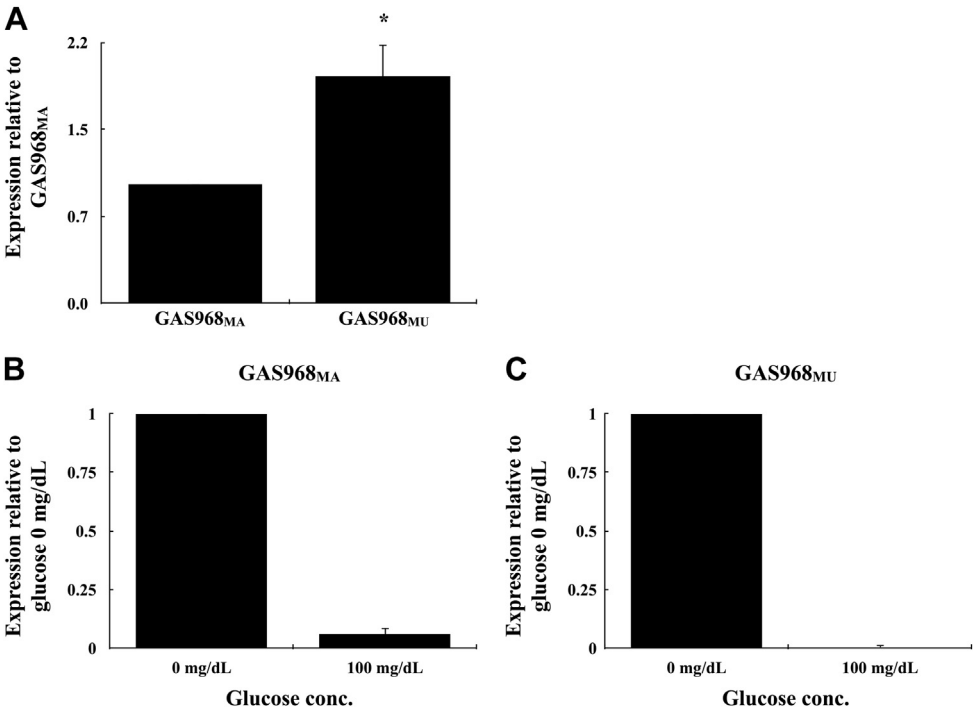

Figure 2. Lactate oxidase gene (*lctO*) expression of mucoid (GAS968$_{MU}$) and hypermucoid (GAS968$_{MA}$) variants. Bacteria were cultured in (A) C medium with agitation for 5 hours, or (B, C) in C medium supplemented with/without glucose for 3 hours, and RNAs were extracted for real-time polymerase chain reaction analysis. Biological replicate experiments were performed from at least three independent RNA preparations in duplicate. Expression level of each target gene was normalized to *gyrA*. *$p < 0.05$.

Carbohydrate metabolic activity is an important indicator to evaluate bacterial fitness under specific conditions. To further clarify whether $GAS968_{MU}$ and $GAS968_{MA}$ have a similar fitness level under aerobic and anaerobic conditions, the glucose utilization and lactate accumulation of these two variants were analyzed. Results showed that $GAS968_{MA}$ significantly utilized glucose more efficiently under the aerobic conditions (Fig. 3B). After 16 hours of incubation, both $GAS968_{MA}$ and $GAS968_{MU}$ utilized more glucose under anaerobic conditions than under aerobic conditions (Table 2). The accumulation of lactate in bacterial culture supernatants under aerobic and anaerobic conditions was also analyzed. Results showed that the lactate concentration in culture supernatants from $GAS968_{MU}$ and $GAS968_{MA}$ showed no difference under both aerobic and anaerobic culture conditions (data not shown).

Table 2 Glucose concentration in bacterial culture supernatants after 16 hours of incubation.

Strain/culture condition	Aerobic condition (A_{500})	Anaerobic condition (A_{500})
$GAS968_{MU}$	0.3208 ± 0.0186	0.0002 ± 0.0001
$GAS968_{MA}$	0.3257 ± 0.0462	0.0079 ± 0.011

C medium supplemented with 100 mg/dL glucose: $A_{500} = 0.423 \pm 0.038$; C medium only: $A_{500} = 0.035 \pm 0.006$.

Discussion

The morphology heterogeneity of GAS is considered to be linked to variations in virulence.[6] Mucoid GAS isolates, which have mutations in two-component regulatory system *covR* or *covS* genes, are associated with invasive manifestations.[10,11] The increase in hyaluronic acid capsule production and DNase (Sda1) expression makes *covR/S* mutants more resistant to immune clearance compared with the wild-type strains.[7] Our previous study showed that hypermucoid GAS is even more virulent than mucoid GAS in the mouse infection model.[15] However, the underlying mechanism of the morphology variation (from mucoid to hypermucoid) is still unknown. In the present study, we showed that oxygen is an important environmental factor that inhibits the morphology conversion of the *emm12*-type GAS968 strain.

Previous studies have shown that GAS can be divided into two groups with respect to the production of hydrogen peroxide.[20,21] In addition, the lactate oxidase activity is almost exclusively responsible for hydrogen peroxide production.[17,20] Our previous study showed that $GAS968_{MA}$ is more sensitive to oxidative stress.[15] The expression of *lctO* in $GAS968_{MU}$ is significantly higher than that of $GAS968_{MA}$ (Fig. 2A), suggesting that $GAS968_{MU}$ might produce more H_2O_2 under aerobic conditions, which may have an important role in the selection of mucoid variants under aerobic conditions. However, there was no detectable H_2O_2 in culture supernatants of $GAS968_{MA}$ and $GAS968_{MU}$ under both aerobic and anaerobic conditions (Table 1). These results suggest that although both $GAS968_{MA}$ and $GAS968_{MU}$ have normal *lctO* RNA transcription, the activity of lactate oxidase in GAS968 may be defective. In addition, hydrogen peroxide is not an important environmental factor for the selection of mucoid variants under aerobic conditions.

Growth under aerobic conditions may produce reactive hydroxyl radicals that can attack bases and deoxyribose residues on nucleotides and lead to bacterial death.[23,24] Our previous study showed that $GAS968_{MA}$ is more sensitive to DNA-damaging conditions.[15] Therefore, the decrease in the number of hypermucoid colonies in the total bacterial population might be caused by growth repression or bacterial death under aerobic conditions. However, the bacterial growth rates in the log to exponential phase under anaerobic conditions of both $GAS968_{MU}$ and $GAS968_{MA}$ were 1.41- to 1.64-fold faster than those cultured under aerobic conditions. In addition, $GAS968_{MA}$ utilized glucose more effectively compared with $GAS968_{MU}$ under aerobic conditions (Fig. 3B). These results suggest that the decrease in the number of hypermucoid variants in the total bacterial population may not be caused by the decrease of its fitness under aerobic conditions.

In the present study, we found that the morphology variation of GAS968 can be manipulated by changing the

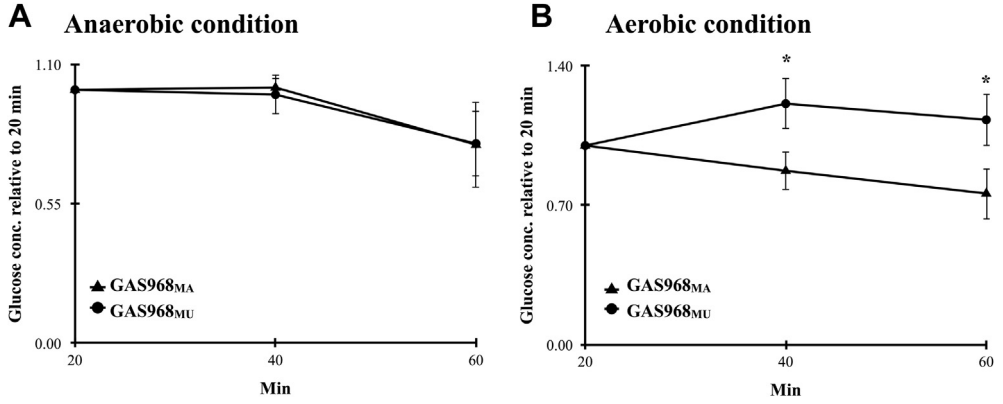

Figure 3. Glucose utilization of mucoid ($GAS968_{MU}$) and hypermucoid ($GAS968_{MA}$) variants under anaerobic or aerobic conditions. Overnight-cultured bacteria were transferred to C medium supplemented with 100 mg/dL glucose, and cultured under (A) anaerobic condition and (B) aerobic condition for 1 hour. Glucose concentration in bacterial supernatant was measured using the spectrophotometric method at A_{500}. *$p < 0.05$.

oxygen concentration in the culture medium. Although the mechanism of morphology variation cannot be defined, our results showed that oxygen is an important environmental signal to inhibit the morphology conversion of GAS968. Morphology conversion is an important factor for increasing GAS968's invasive activity in the mouse infection model. The oxygen-dependent morphology variation property of GAS968 may have important roles in its pathogenesis.

Conflicts of interest

The authors affirm no conflict of interest relative to any source of funding, sponsorship, or financial benefit.

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