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ORIGINAL ARTICLE

# Oxygen-dependent phenotypic variation in group A streptococcus

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**KEYWORDS**
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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