SPANDIDOS PUBLICATIONS

# Fever temperature enhances mechanisms of survival of *Streptococcus agalactiae* within human endothelial cells

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**Abstract.** Group B *streptococci* (GBS) are the most common cause of pneumonia and sepsis during the neonatal period. However, the pathogenesis of invasive infection is poorly understood. We investigated the ability of GBS grown at 37˚C and 40˚C to adhere and invade human umbilical vein endothelial cells (HUVECs) at different periods of incubation (0, 0.5, 1, 2, 18 and 24 h). All strains tested, except strain 88641-vagina survived for 24 h in the intracellular environment at 40˚C. For serotype III grown at 40˚C, both strains (80340-vagina and 90356-liquor) showed increased adherence and intracellular survival when compared to bacteria grown at 37˚C (P<0.01). GBS serotype V strains (88641-vagina and 90186-blood) showed ability to survive inside HUVECs until 2 and 24 h post-infection at 40˚C and 37˚C, respectively (P<0.01). Influence of growth temperature in bacterial interaction with endothelial cells was partially dependent of serotypes and the clinical origin of strains. Serotypes III and V strains grown at both temperatures remained viable within acidic endothelial vacuoles which acquired Rab7 and LAMP-1 endosomal markers. The data emphasize the influence of temperature on cellular events of phagocytosis and pathogenesis of GBS diseases.

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## Introduction

*Streptococcus agalactiae*, also known as group B *streptococcus* (GBS), is a commensal inhabitant of the human gastrointestinal and genitourinary tract. Pregnant women who carry GBS asymptomatically can transmit bacteria to their newborns, sometimes resulting in severe neonatal infection. Intrapartum maternal risk factors such as fever, prolonged rupture of membranes, and chorioamnionitis can determine if asymptomatic neonates would develop a neonatal sepsis right after delivery (1). Intrapartum temperature >38˚C is associated with an increased risk of neonatal GBS infection. Studies describe a greater probability of intrapartum fever in mothers of infants with confirmed GBS infection (2).

The modulation of virulence in response to environmental changes is a common trait of pathogenic bacteria (3). GBS can monitor the environment and alter gene expression in response to many factors, including temperature changes. Bacterial incubation at temperatures closer to that occurring in patients with severe infection and high fever (40˚C) induced differences in GBS genes implicated in virulence, as the up-regulation of several transcripts. For example, the genes in the hemolysin operon cyl, CAMP factor (an extracellular cytolytic protein involved in GBS pathogenesis), proteins with LPXTG motifs, chaperones, heat-shock proteins and a heat-inducible transcription regulator were clearly overexpressed at 40˚C (4,5).

The development of GBS disease reflects successful bacterial colonization of the vaginal epithelium, penetration of placental or epithelial barriers, resistance to immune clearance allowing bloodstream survival (6). The clinical scenario concerning invasive diseases caused by GBS underlies the ability of this pathogen to bypass and/or injure endothelial cells to gain access to the blood stream. Previous studies demonstrated that the GBS-endothelial cell interaction process is a phenomenon of prime importance in the etiology of diseases caused by GBS, but an understanding of these events at the

molecular level is still incomplete in the early stage of characterization (7,8). Many factors that allow this microorganism to persist and thrive in human tissues remain unidentified.

To better understand the GBS disease process, we have studied the interaction of GBS serotypes III and V with human endothelial cells under similar conditions as human physiological temperature (37˚C) and fever conditions (40˚C).

## Material and methods

*Bacterial strains*. GBS serotype III (strains 80340-vagina and 90356-liquor) and serotype V (strains 88641-vagina and 90186-blood), were used in this study and partially investigated for adhesive properties (9). The strains were identified as group B and serotyped as described previously (Lancefield, 1934). The microorganisms were stored after lyophilization and recovered in Brain Heart Infusion broth (BHI; Difco Laboratories, Detroit, MI). GBS isolates were cultured on Blood Agar base (Oxoid) plates containing 5% sheep desfibrinated blood (BAB/blood) for 24 h at 37˚C and then grown in BHI (Difco Laboratories) at temperatures of 37˚C and 40˚C until 0.4 OD (~$10^8$ CFU $ml^{-1}$) (10).

*Human umbilical vein endothelial cell (HUVECs) culture*. Primary HUVECs were routinely obtained by treating umbilical veins with 0.1% collagenase IV solution (Sigma Chemical Co., St. Louis, MO, USA) as previously described (11) and maintained in 199 medium (M199)/HEPES modification (Sigma) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 $\mu$g/ml and anphotericin-B 2.5 $\mu$g/ml), glutamine 2 mM and 20% fetal calf serum (FCS, Cultilab, Campinas, Brazil), at 37˚C in a humidified 5% $CO_2$ atmosphere until they reached confluence. Cells were used in first or second passages only, and subcultures were obtained by treating the confluent cultures with 0.025% trypsine/0.2% EDTA solution in PBS (9).

*Adherence and internalization assays*. Confluent cultures of HUVECs were allowed to interact with GBS ($5x10^7$ CFU) submitted to growth temperatures of 37˚C or 40˚C. For the bacterial binding assays, infected monolayers were incubated for 0, 0.5, 1, 2, 18 and 24 h in 5% $CO_2$ at 37˚C. After each incubation period, infected cells were rinsed three times with M199, and then lysed in a solution of 0.5 ml of 25 mM Tris, 5 mM EDTA, 150 mM NaCl plus 1% Igepal. Total GBS association (intracellular plus surface adherent) was estimated by plating endothelial lysates and counting the resulting colonies that appeared in BAB plates containing 5% sheep desfibrinated blood. To measure bacterial internalization, a similar assay used for adherence was performed. After each period of incubation, infected monolayers were rinsed three times with M199, and incubated for an additional 2 h period in M199 containing bactericidal amounts of both gentamicin (100 $\mu$g $ml^{-1}$) and penicillin G (5 $\mu$g $ml^{-1}$). The number of internalized bacteria was determined as outlined above. The adherence rates were determined as follows: [CFU of total cell-associated (intracellular viable plus surface adherent) GBS - CFU intracellular GBS] (9).

*Analysis of the acidic compartments of endothelial cells by orange acridine labelling*. Acidification of bacteria-containing phagocytic vacuoles was observed in HUVECs treated with orange acridine and analyzed by laser confocal microscopy. Endothelial cells were infected for 2 h at 37˚C with GBS grown to 37˚C or 40˚C. Subsequently, infected HUVECs were washed and treated with 200 $\mu$l M199 with orange acridine, re-incubated for 20 min at 37˚C/5% $CO_2$ and washed with M199 without orange acridine. The presence of orange-stained GBS was considered indicative of the GBS-containing vacuole acidification (confocal laser scanning microscopy (CLSM Zeiss 310) (12).

*Endocytic markers on maturing GBS-containing vacuoles (GBS-CV)*. For determining lysosomal-endocytic pathway compartments involved in the internalization of GBS, double-marking by anti-Rab7 and anti-LAMP-1 were performed after 1 h incubation with bacterium grown at 37˚C and 40˚C. Subsequently, cells were previously fixed at 3.5% formaldehyde and incubated with PGN (PBS, 0.15% gelatin, 0.1% sodium azide) with 0.1% of saponin for 30 min. After, infected HUVECs were initially stained with monoclonal antibody (MAbs) anti-Rab7 (Santa Cruz), 1:50 diluted in PGN/0.1% of saponin for 1 h and then coverslips were incubated with conjugate anti-rabbit IgG (Santa Cruz). Then, cells were incubated with monoclonal antibody anti LAMP-1 (supernatant of the clone H4A4 anti-human LAMP-1, developed in mice, Developmental Studies Hybridoma Bank, IA, USA) for 1 h. Finally, the material was incubated for 1 h with DAPI (10 mM, Sigma) and in conjunction with anti-mouse IgG-CY3 (Sigma) diluted 1:50 in PGN/saponin. Images of Rab7 were acquired by Nikon E600 microscope with Nikon DXM1200 digital Camera using ACT-1 software. Adobe Photoshop was used to pseudocolor the images. Fluorescence images of MAbs were generated in green and DNA labeling is shown in red and imaged LAMP-1 using confocal laser microscopy using a Zeiss Axiovert 100 microscope with a x100 Plan-apochromatic 1.4 oil immersion objective attached to an MRC-10240V confocal system (BioRad, Hercules, CA, USA) (13).

*Statistics*. All data are expressed as means ± standard deviations (error bars). Data were analyzed by two-tailed t-test. A P-value of $<0.05$ was considered significant.

## Results

*GBS adherence and internalization in HUVECs*. Adherence and intracellular viability data expressed as CFU/ml are shown in Fig. 1. For serotype III grown at 40˚C, both strains showed higher ability of adherence and intracellular survival when compared to bacteria grown at 37˚C ($P<0.01$; Fig. 1A-D). For the vagina isolate 80340-III, intracellular viable bacteria at 1, 2 and 24 h were observed only when microorganisms were grown at 40˚C. Conversely, GBS serotype V strains showed similar ability to adhere and survive inside HUVECs cells after growth at 37˚C or 40˚C ($P<0.01$; Fig. 1E-H). The vagina 88641-V strain was the only isolate unable to survive for 24 h in the intracellular compartment at 40˚C (Fig. 1F). Therefore, the influence of growth temperature in bacterial interaction with endothelial cells was partially dependent of serotypes and of the clinical origin. For vagina isolates of serotypes III and V the increase of temperature (40˚C) favored

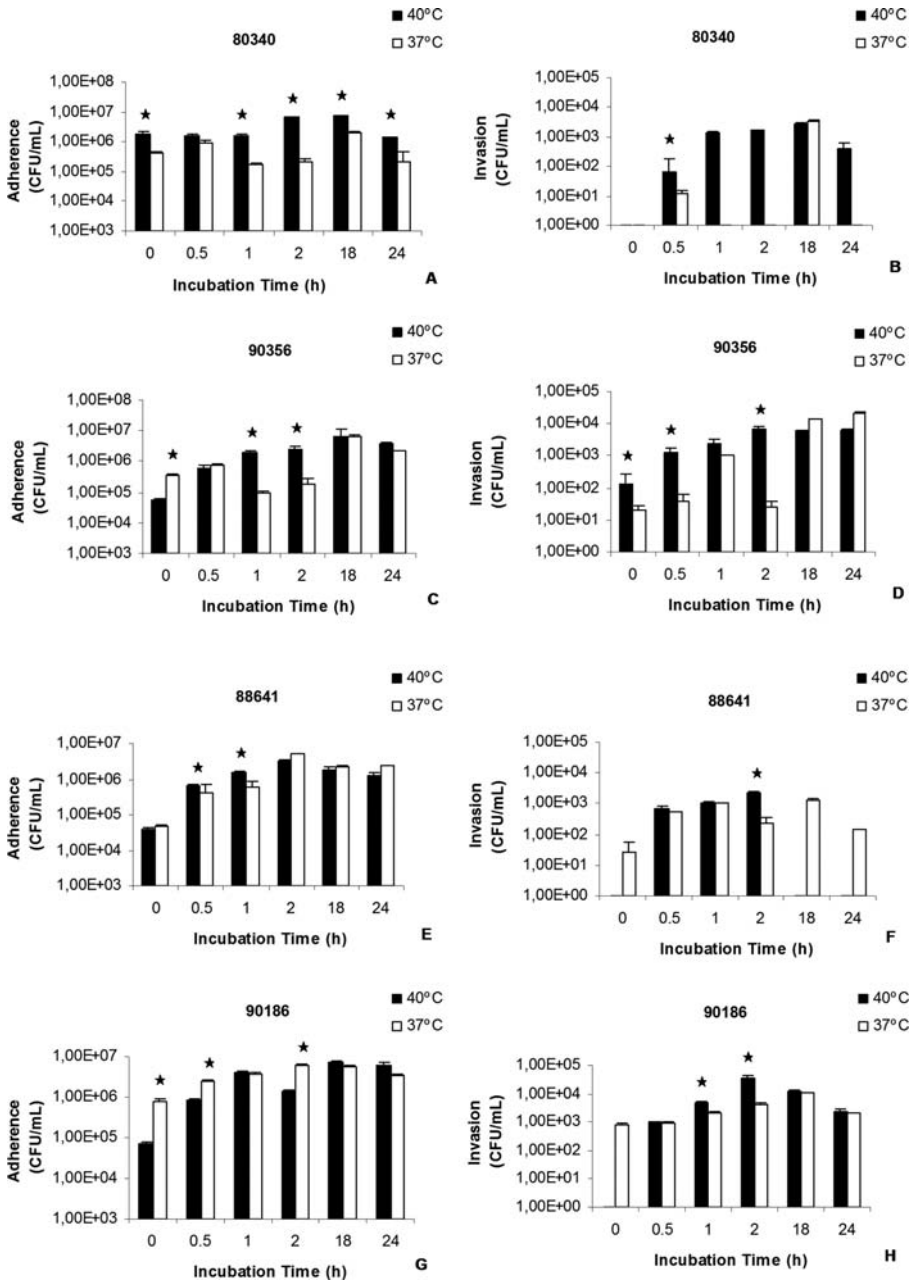


Figure 1. Adherence (A, C, E and G) and intracellular viability (B, D, F and H) of GBS isolates to endothelial cells (HUVECs), 80340 and 90356 (serotype III; vagina and liquor), and 88641 and 90186 (serotype V; vagina and blood). Each value is the mean ± SEM of four samples. Black, GBS grown at 40˚C; white, GBS grown at 37˚C.

survival into HUVECs only for the 80340-III strain (Fig. 1B; P<0.001). Invasive strains isolated from CSF (90356-III) and blood (90186-V) showed survival ability within HUVECs until 24 h incubation for both temperatures (Fig. 1D and H).

*GBS-CV acidification in HUVECs*. Orange acridine labelling assays showed the formation of acidic compartments into GBS-containing vacuoles inside HUVECs (Fig. 2A-C) independent of serotypes and growth temperature.

*GBS-CV acquire markers of late endosomes/lysosomes during maturation*. Confocal microscopy assays demonstrated that GBS-CVs were enriched in LAMP-1 at 1 h post-infection for the blood isolate (90186-V strain) grown at both temperatures (37˚C and 40˚C) as illustrated in Fig. 2D-F. Vacuolar recruitment of Rab7 during intermediate stages of GBS-CV maturation was also detected (Fig. 2G-I).

## Discussion

Sudden temperature upshift may induce temporary growth inhibition of bacterial cells and rearrangement of gene expression directed towards survival, including for GBS strains (5,14-16). The present study demonstrated that temperature changes may influence GBS survival within endothelial cells. For serotype III, the adherence to and internalization by HUVECs were up-regulated at 40˚C relative to 37˚C, suggesting that GBS undergoes an adaptive response of growth to increased temperature. Conversely, reduction in the number of intracellular viable bacteria in response to a temperature

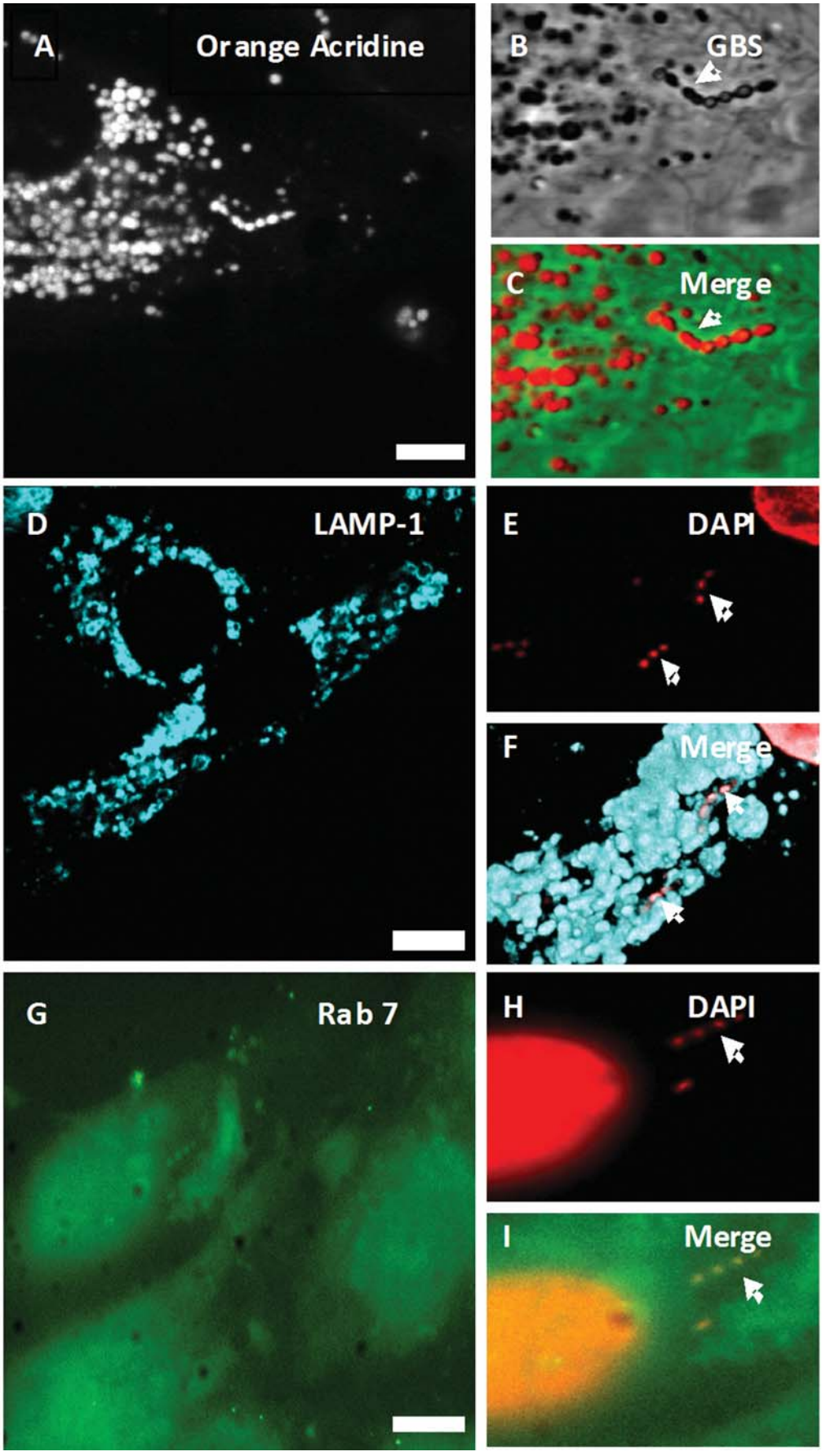


Figure 2. Acidification and maturation of GBS-containing vacuoles inside endothelial cells (HUVECs). Photomicrographs of acidic compartments of endothelial cells submitted to fluorescence orange acridine labelling showing the acidification of GBS-containing vacuoles inside HUVECs (A-C) after 1 h incubation with bacteria. Lysosomal-endocitic pathway compartments involved in the internalization of GBS, double-marked by anti LAMP-1 (D-F) and anti RAB-7 (G-I) were performed after 1 h incubation with DAPI-labeled GBS grown at 40˚C. Arrows indicate GBS in whole and close-up images. Scale bar is 20 $\mu$m.

om 37˚C to 40˚C was observed 2 h post-infection rotype V strains. Such results underscore the great complexity of the bacterial stress response induced by growth temperature, with mechanisms involving proteins required during bacterial growth, whereas others are only necessary during a specific step of the growth (5).

In contrast to GAS, relatively little is known about the function of putative extracellular proteins expressed by GBS. Many of these proteins may participate in adherence to host molecules during colonization or invasion. It has been shown previously that there was no significant difference in transcript levels between 30˚C and 40˚C for genes involved in the synthesis of the two known pilin systems in GBS (5). In contrast, several other genes encoding putative cell-surface proteins with an LPXTG/LPXTN cell-wall anchoring motif were differentially expressed according to the temperature. Interestingly, protein involved in adherence, protein which cleaves the complement component C5a and mediates adherence to fibronectin, hemolysin production and protein which has been implicated in resistance to phagocytic killing and GBS survival in human blood, were up-regulated at 40˚C (5,17-19). Our data suggest that potentially invasive GBS strains isolated from vagina, cerebrospinal fluid and blood express surface molecules induced by exposure to fever temperatures. These proteins up-regulated by temperature may be required for adherence during pathogenesis (colonizing the lungs of neonates) and invasion (blood, cerebrospinal fluid), thus providing distinct advantages to some GBS strains during the initial steps of infection.

Intravacuolar pathogens have devised various strategies to avoid degradation along the endocytic pathway (20). Some of them avoid interactions with the endocytic pathway, like *Legionella pneumophila* and *Chlamydia* species (21). Some species arrest the maturation of their phagosome at various stages, such as *Mycobacterium tuberculosis* (20). Ultimately they avoid fusion with degradative lysosomes. The key process in the maturation of a newly formed phagosome to a degradative phagolysosome is its progressive acidification through the sequential interactions with compartments of the endocytic pathway (22). Our results indicated that both mechanisms of inhibition of phagosomal acidification and lysosomal fusion were not involved in the survival of GBS strains into HUVECs, independent of their growth temperature.

In addition our data showed the transient accumulation of the late endosomal/lysosomal marker LAMP-1 on maturing GBS-CV for bacteria grown at 37˚C or 40˚C. Moreover, we also showed GBS-CV recruitment of Rab7, demonstrating that GBS-CV traffic along the endocytic pathway and fuse with lysosomes. These data demonstrating the interaction of intermediate GBS-CV with the late endosomal/lysosomal compartment suggest that GBS is capable of resisting a potentially degradative environment, at least transiently. Similarly, opsonized *Brucella abortus* (23) and *Staphylococcus aureus* (24) remained viable within LAMP-1-positive compartment in human monocytes and endothelial cells, respectively.

In conclusion, this work reports the influence of temperature on the bacterial growth and the process of interaction of GBS with human endothelial cells. We have shown for the first time the acquisition of the endosomal markers in endothelial GBS-CV. Temperature induced alterations during infection of HUVECs by GBS, especially serotype III that contributes to its survival in the blood and possible dissemination to other anatomic sites.

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