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The CovS/CovR Acid Response Regulator Is Required for Intracellular Survival of Group B *Streptococcus* in Macrophages

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Group B *Streptococcus* (GBS) is a leading cause of neonatal meningitis and septicemia. The ability of this organism to survive inside phagocytic cells is poorly understood but thought to be an important step for the establishment of disease in the host. Here, we demonstrate that GBS shows prolonged survival within J774 macrophages and that the capacity to survive is not significantly changed across a diverse range of strains representing different serotypes, multilocus sequence types (MLST), and sites of clinical isolation. Using staining for the lysosome-associated membrane protein (LAMP) and by pharmacological inhibition of phagosome acidification, we demonstrate that streptococci reside in a phagosome and that acidification of the phagosome is required for GBS to survive intracellularly. Moreover, we show that the GBS two-component system CovS/CovR, which is the major acid response regulator in this organism, is required for survival inside the phagosome.

Lancefield group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is a Gram-positive encapsulated bacterium exhibiting β-hemolysis on blood agar. The organism is found as a commensal in the gastrointestinal and the genitourinary tracts of up to 30% of healthy adults (3). However, GBS is a significant cause of neonatal meningitis and septicemia; in 2009, the rate of bacteremia in United Kingdom neonates up to 90 days old was 0.64 per 1,000 live births (20). Infection is seen increasingly in adults, especially those with underlying diseases such as diabetes mellitus, and in 2005 two-thirds of all invasive GBS infectious in the United States were in older adults (14). Group B streptococci are subclassified into 10 serotypes according to the immunological reactivity of the polysaccharide capsule. Serotypes Ia, Ib, II, III, and V are responsible for the majority of human invasive disease (51). Serotype III is the serotype most often isolated from both early- and late-onset neonatal disease and accounts for 80% of cases of neonatal meningitis. Serotype V is the most common serotype associated with GBS-infected nonpregnant adults (18, 24, 51). Within serotype III, multilocus sequence typing (MLST) has identified a hypervirulent lineage termed ST-17, which is more likely to be recovered from meningitis than other serotype III strains; this lineage appears to be a highly successful invasive clone (4, 34, 40).

The ability of GBS to remain with the host as a commensal organism and to establish infection in susceptible individuals suggests that the organism may be able to subvert the host immune system. It is known that when unopsonized group B streptococci are engulfed by professional phagocytic cells, such as macrophages and neutrophils, the organism can remain viable for several hours (12, 31, 49), although the mechanism of survival is unknown. The intracellular localization of GBS in macrophages may protect the organism from more active antimicrobial molecules in the blood and thus may be important in establishing bacteremia and subsequent meningitis. In line with this, in the closely related organism and extracellular pathogen *Streptococcus pyogenes*, it has been suggested that an important mechanism to establish infection is the survival of the organism in phagocytic cells (25).

Recognition of an organism by the phagocytic cells of the innate immune system leads to engulfment of the organism into a phagosome, which matures through fusion with membrane-bound endosomes and lysosomes to form the phagolysosome. Very quickly (within 15 min) after engulfment, the environment in the lumen of the phagosome becomes acidified through the fusion of early endosomes (acidified endocytic vesicles) and the activity of V-type ATPases. Maturation to the late phagosome is characterized by an increasingly acidic lumenal pH and the presence of proteases and lysosome-associated membrane proteins (LAMPs). The fusion of the phagosome with lysosomes, organelles containing hydrolytic enzymes, results in the formation of a phagolysosome and the culmination of the maturation process. The phagolysosome is a hostile environment for the microorganism, since it is highly acidic and contains reactive oxygen species (ROS), reactive nitrogen species (RNS), and antimicrobial proteins and peptides (1, 15).

In the present study, we have characterized the interaction between the phagosome and the ingested group B *Streptococcus* in order to understand the molecular basis of the prolonged intracellular survival of GBS. Using a collection of GBS strains isolated from different clinical presentations and representing a range of serotypes and MLSTs, we have demonstrated that all strains have the ability to survive for a period intracellularly, suggesting that all strains have the potential to cause disease. We have also studied deletion mutants in major virulence factors, such as the polysaccharide capsule (42) and the CylE operon (31), as well as strains with the two-component system (TCS) CovS/CovR (alternatively named CsrS/R) disrupted (22, 28). We show that GBS-containing phagosomes accrue markers of phagosomal maturation and that phagosomal acidification is required to support prolonged intra-

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cellular survival of GBS. In addition, while several known GBS virulence factors are dispensable for intracellular life, the CovS/CovR system is critical for survival within the phagosome.

MATERIALS AND METHODS

Bacterial culture. Bacterial strains used are listed in Table 1. Group B streptococcal strains were grown in static culture at 37°C in THY broth consisting of Todd Hewitt broth (Sigma) with the addition of 5% yeast extract (MP Biomedicals). *Lactococcus lactis* was grown in static culture at 37°C in M17 broth (Sigma) with 0.5% glucose and on M17 agar plates at 28°C. *Escherichia coli* was grown in shaking culture at 37°C in LB broth. Where required, GBS was heat killed at 60°C for 30 min.

J774 cell line. The J774.16 mouse monocyte-derived macrophage-like cell line was cultured in Dulbecco's modified Eagle medium (DMEM; Sigma) with the addition of 2 mM L-glutamine, 10,000 U penicillin (Sigma), 10 mg/ml streptomycin (Sigma), and 10% fetal bovine serum (FBS; GIBCO), here referred to as "complete DMEM." Cells were incubated at 37°C in 5% CO_2. The cell line was used from between five and 15 passages after thawing.

Survival assay. J774 cells were plated into either a 24-well tissue culture dish at a concentration of 1×10^5 macrophages/well in 1 ml of complete DMEM or a 6-well tissue culture-treated plate at 3×10^5 in 3 ml of complete DMEM. Cells were left to settle for 16 to 24 h before the assay. Organisms used in the assay were grown overnight, washed twice in warm 1× phosphate-buffered saline (PBS), and diluted as appropriate in serum-free DMEM (DMEM with the addition of glutamine but not antibiotics or FBS). Before infection, macrophages were washed three times in warm PBS to remove the complete DMEM and antibiotics, and the medium was replaced with serum-free DMEM containing organisms at a ratio of approximately 10 bacterial cells to one macrophage (multiplicity of infection [MOI] is 10:1). Bacteria were allowed to internalize for 30 min at 37°C in 5% CO_2. Extracellular bacteria were removed by washing macrophages three times in PBS, and the medium was replaced with serum-free DMEM containing 5 μg/ml penicillin and 100 μg/ml gentamicin; the cells remained in this medium for the assay. Cells were cultured throughout all experiments at 37°C in 5% CO_2.

To measure the rate of survival of the intracellular bacteria, samples were taken 30 min after the addition of antibiotics (time zero) and then at 3, 6, 9, and 12 h after this. To estimate the number of surviving intracellular microorganisms, infected cells were washed three times with PBS and then incubated for 10 min at 37°C with 0.02% Triton-X, to lyse the macrophages and release intracellular bacteria (this concentration of Triton-X does not affect bacterial survival). The lysate was then removed, the bacterial cells were spun down, and the pellet was resuspended in PBS. The bacterial cell suspension was diluted and plated out onto agar plates, and visible colonies were counted after 22 h of incubation to give a count of CFU. To analyze the number of bacteria surviving over time compared to the initial number of intracellular bacteria, the relative CFU (rCFU) was calculated as follows: rCFU = CFU at time point *x*/CFU at time point 0.

The assay was repeated several times, and the data were analyzed using SPSS 16.0 software. The Mann-Whitney U test or Kruskal Wallis test were used with a cutoff *P* value of <0.05 to establish differences between strains or conditions. Results are presented as box and whisker plots; the box represents the upper and lower quartiles, with the line in the center the medium value of all repeats, and the whiskers are drawn to the maximum and minimum values, excluding outliers (marked with open circles), which are between 1.5 and 3 box lengths away from the ends of the plot, and extreme values (marked with asterisks), which are over 3 box lengths away from the end of the plot.

Drug treatment. For assays looking into the effect of concanamycin A, 0.1 μM concanamycin A (Calbiochem) dissolved in dimethyl sulfoxide (DMSO) was added to the serum-free DMEM when the antibiotics were added and remained with the cells for the assay time. To investigate the effect of iron supplementation, compounds were made as previously described (2). Ferric chloride (FeCl) was dissolved in distilled water (dH_2O) with 2% HCl to a concentration of 6 mg/ml, nitrilotriacetate (NTA) was dissolved in dH_2O, and the pH was adjusted to neutral using NaOH. Both preparations were filter sterilized. To make up ferric nitrilotriacetate (FeNTA), the FeCl and NTA were mixed at a 1:1 ratio (3 mg/ml), and the pH was adjusted to neutral by the addition of 0.9 M NaOH. The volume of NaOH added was recorded to allow for an estimation of the concentration of FeNTA. The solution was then filter sterilized through a 0.22-μM-pore-size filter. All solutions were made fresh for each experiment. Iron solutions were then added to GBS-infected J774 cells at time zero (T_0) at concentrations of 5 μM, and 50 μM FeNTA and 50 μg/ml of NTA or FeCl remained in the medium for the duration of the experiment.

Measurement and inhibition of reactive oxygen species. To measure generation of reactive oxygen intermediates in infected and noninfected J774 cells, we used the cell permanent fluorescent dye 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). J774 cells removed from plastic using Accutase (Sigma) were washed twice in PBS and then incubated with 5 μM H_2DCFDA in PBS at 37°C for 20 min. Cells were then analyzed by flow cytometry using a FACSCaliber instrument and analyzed with CellQuestPro (BD Biosciences). A total of 10,000 events were counted, and the percentage of positive cells was identified by gate analysis.

Production of reactive oxygen species was inhibited in macrophages using the NADPH oxidase inhibitor apocynin (4-hydroxy-3-methoxyacetophenone). Apocynin was dissolved in serum-free DMEM overnight at 50°C to make a stock solution of 15 mM, which was added to cells at the time of infection at a final concentration of 0.5 mM (21). Apocynin remained in the medium for the duration of the experiment.

Sensitivity of GBS strains to physiological stress. To asses the organisms' resistance to the conditions of physiological stress that would be experienced in the phagosome, an overnight culture of GBS was washed in PBS and then diluted 1/500 into buffer containing the stressor; this gave an inoculum of GBS of approximately 1×10^6 organisms/ml. To investigate nitrosative stress, sodium nitrite ($NaNO_2$) was added at concentrations from 0.25 to 20 mM (50) in a 0.1 M sodium phosphate buffer at pH 4.4. Sensitivity to reactive oxygen intermediates was measured by adding hydrogen peroxide (H_2O_2) to 0.1 M sodium phosphate buffer at pH 7.4 at concentrations from 0.125 mM to 14 mM (50). After 1 h of incubation at 37°C, cultures were diluted and plated onto THY plates to give an estimated CFU/ml count. To test acid tolerance, approximately 1×10^6 organisms/ml were added to 0.1 M sodium phosphate buffers of various pHs and incubated at 37°C for 1 h. Viable counts were estimated by serial dilution and plating at the end of this incubation period and compared with the number of organisms initially. Sodium phosphate buffers of specified pHs were made by mixing 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 and adjusted with HCl when necessary.

Immunofluorescence. To stain for lysosome-associated membrane protein (LAMP), the supernatant of the rat monoclonal antibody ID4B was used at a 1/25 dilution. (Developed by J. Thomas August, ID4B was obtained from the Developmental Studies Hybridoma Bank and developed under the auspices of the NICHD and maintained by the University of Iowa.) To stain for GBS, the mouse anti-*Streptococcus agalactiae* monoclonal 1.B.501 antibody was used diluted 1/50 (Santa Cruz Biotechnology). Secondary antibodies used were anti-rat tetramethyl rhodamine isocyanate (TRITC) conjugate for the detection of LAMP and anti-mouse fluorescein isothiocyanate (FITC) conjugate for the detection of GBS, both at a concentration of 1/100 (Invitrogen).

J774 cells were seeded overnight on 12-mm coverslips and then infected with GBS, heat-killed GBS, or latex beads. Infection was synchronized by incubating GBS or beads with J774 cells on ice for 30 min; during this time and under these conditions, adherence will take place but phagocytosis will be inhibited. The assay plates were moved to 37°C to stimulate phagocytosis, and the experiment was carried out as described above for the survival assay except for the procedure at each time point. At specific time points, the coverslips were removed and fixed for 10 min at room temperature in 4% paraformaldehyde (wt/vol in PBS). Coverslips were washed three times in PBS and quenched for 10 min in 50 mM ammo-

TABLE 1 Bacterial strains used in this study

Strain	Genotype	Relevant characteristics (reference[s])[a]
Group B streptococcal strains		
NEM316	GBS wild type	Serotype III isolated from neonatal blood culture (early-onset disease), ATCC 12403, CIP82.45
A909	GBS wild type	Type Ia mouse-passaged prototype strain 090, Isolated from neonatal blood culture (36)
COH1	GBS wild type	Highly encapsulated type III strain, isolated from neonatal blood culture, ST-17 (42)
2603v/r	GBS wild type	Serotype V, ST-110 (47)
K1		Serotype III, ST-17, LOD (4, 19)
M781		Serotype III (47)
M732		Serotype III (47)
H7783		
COH31	GBS wild type	Serotype III, ST-17
GBS6313	GBS wild type	Serotype III
z50	Clinical isolate	Serotype III, ST-19, colonizing (19)
j76	Clinical isolate	Serotype III, LOD (19)
j95	Clinical isolate	Serotype III, ST-17, LOD (4, 19)
m1	Clinical isolate	Serotype III, LOD (19)
mk2	Clinical isolate	Serotype III, ST-17, LOD (4, 19)
b9	Clinical isolate	Serotype III, ST-17, EOD (4, 19)
mk3	Clinical isolate	Serotype NT, EOD (19)
b11	Clinical isolate	Serotype III, LOD (19)
j99	Clinical isolate	Serotype Ia, EOD (19)
b3	Clinical isolate	Serotype V, EOD (19)
z77a	Clinical isolate	Serotype II, ST-19, colonizing (19)
z34a	Clinical isolate	Serotype III, ST-17, colonizing (19)
z37	Clinical isolate	Serotype III, ST-17, colonizing (19)
z18a	Clinical isolate	Serotype Ia, ST-24, colonizing (19)
z84a	Clinical isolate	Serotype V, ST-1, colonizing (19)
j87	Clinical isolate	Serotype II, LOD (19)
h11	Clinical isolate	Serotype III, ST-17, EOD (4, 19)
j100	Clinical isolate	Serotype III, ST-17 EOD (4, 19)
j88	Clinical isolate	Serotype III, ST-17, EOD (4, 19)
j96	Clinical isolate	Serotype Ib, EOD (19)
wc3	Clinical isolate	Serotype III, ST-17 EOD (4, 19)
z111	Clinical isolate	Serotype Ib, ST-8, colonizing (19)
z95	Clinical isolate	Serotype V, ST-1, colonizing (19)
z81a	Clinical isolate	Serotype Ia, ST-23, colonizing (19)
z117	Clinical isolate	Serotype III, ST-19, colonizing (19)
z101a	Clinical isolate	Serotype III, ST-19, colonizing (19)
z12a	Clinical isolate	Serotype V, ST-1, colonizing (19)
z41	Clinical isolate	Serotype NT, ST-10, colonizing (19)
z72a	Clinical isolate	Serotype Ib, ST-8, colonizing (19)
j90	Clinical isolate	Serotype III, LOD (19)
z78a	Clinical isolate	Serotype Ib, ST-6, colonizing (19)
j61	Clinical isolate	Serotype Ia, EOD (19)
j81	Clinical isolate	Serotype III, ST-17, EOD (4, 19)
z87a	Clinical isolate	Serotype V, ST-23, colonizing (19)
r1	Clinical isolate	Serotype III, ST-17, EOD (4, 19)
k9	Clinical isolate	Serotype III, LOD (19)
z69a	Clinical isolate	Serotype Ib, ST-12, colonizing (19)
z73	Clinical isolate	Serotype Ib, ST-10, colonizing (19)
Group B streptococcal mutants		
NEM2456	GBS NEM316 Δ*cylE*	In-frame deletion of the *cylE* gene in parental strain NEM316 (16)
NEM2089	GBS NEM316, Δ*covS/R*	NEM316 with the CovS/CovR two-component regulator deleted (28)
COH1-13	*cpsE* disrupted	Tn*917* delta E insertion in *cpsE* (42)
2603*csrR*Δ	2603v/r deleted in *csrR*	In-frame deletion of *csrR* (otherwise called *covR*) (22)
2603*csrR*ΔRep	2603v/r deleted in *csrR* with pJRS233 plasmid carrying wild-type allele	In-frame deletion of *csrR* (otherwise called *covR*) with plasmid repair (22)
Control organisms		
Lactococcus lactis subsp. *cremoris*	MG1363, type strain	Plant and dairy bacterium
E. coli	dH5α	Cloning strain

[a] EOD, early onset disease; LOD, late onset disease.

nium chloride, washed again three times in PBS, and permeabilized using 0.1% Saponin in blocking buffer (PBS with the addition of 3% goat serum). Coverslips were transferred directly to primary antibody (diluted in blocking buffer with the addition of 5× human IgG [Invitrogen]) for 20 min. After primary incubation, coverslips were washed three times and transferred to the secondary antibody (1/100 dilution in blocking buffer with 5× human IgG) for 20 min. This was carried out first using anti-*Streptococcus* primary antibody and then repeated to stain for LAMP. After being labeled, coverslips were washed three times in PBS and three times in water and mounted in Mowiol (Calbiochem, Nottingham, United Kingdom) with the antifade reagent p-phenylene diamine (Sigma). All labeling incubations were at room temperature.

For fluorescence microscopy, cells were viewed under a 100× differential interference contrast (DIC) oil immersion objective on a Nikon

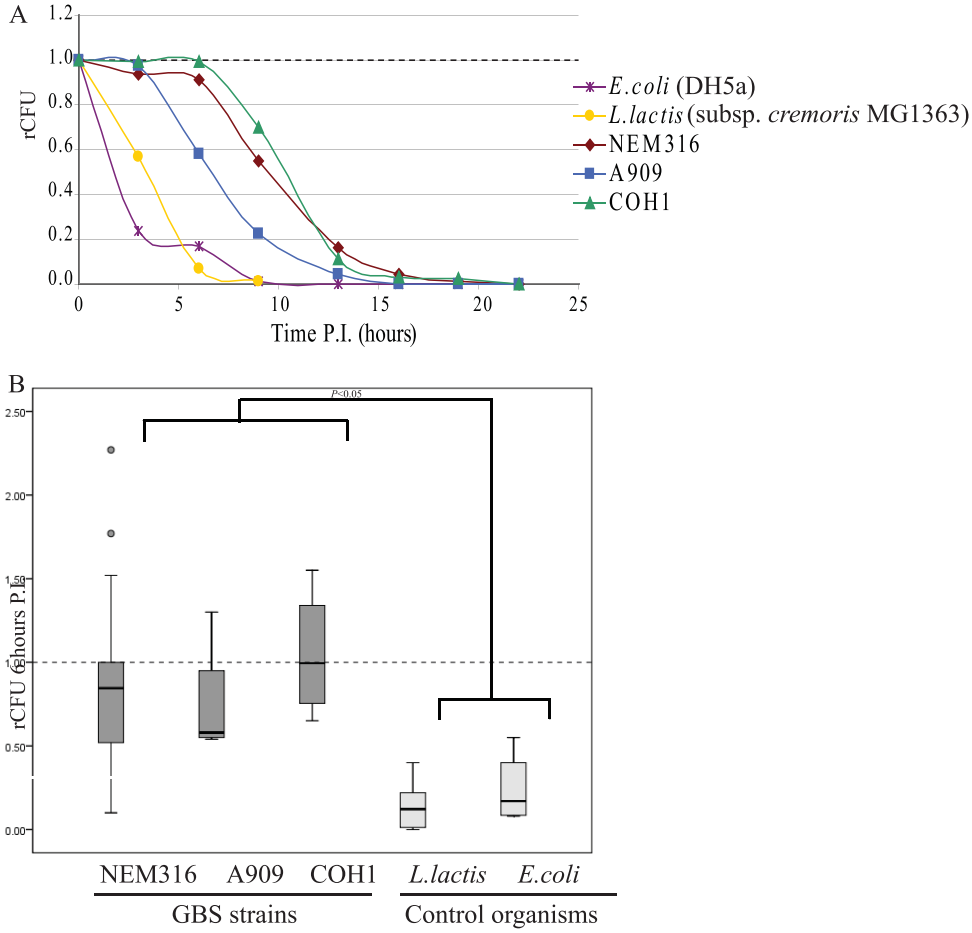


FIG 1 The intracellular survival of three strains of group B *Streptococcus* in J774 macrophages, compared to the nonpathogenic organisms *Lactococcus lactis* (MG1363) and *E. coli* dH5α over a period of 24 h (A) and at 6 h postinfection (P.I.) (B). The relative number of CFU (rCFU) was estimated by plating out the lysate of infected macrophages and counting the number of CFU at each time point. The rCFU is the difference between the initial number of intracellular bacteria and the number at each time point. The three different strains of GBS show no difference between each other but have CFU counts that are all significantly higher than those of both control organisms. $P < 0.05$, Mann-Whitney U test, n (number of replicates) > 5 for each.

Eclipse Ti microscope. Images were captured using an ORCA-R^2 camera (Hamamatsu) and NIS elements software (Nikon). Images were processed in Adobe Photoshop (Adobe), and each phagosome was scored by eye for LAMP acquisition.

RESULTS

Group B *Streptococcus* shows extended survival in J774 macrophages. It has been previously reported that group B *Streptococcus* can survive inside phagocytic cells for longer than nonpathogenic organisms, such as *E. coli* (12, 49). To allow further investigation of the mechanism behind this phenotype, we developed an antibiotic exclusion and survival assay in the J774 macrophage-like murine cell line. J774 cells are considered a useful model cell for macrophage activity and have been used for investigating intracellular survival of many pathogens, including GBS (31, 41, 49).

Although *Lactococcus lactis*, a nonpathogenic species that is closely related to the streptococci, is rapidly killed in J774 macrophages, GBS showed extended survival for up to 12 h in macrophages (Fig. 1). Both GBS and *Lactococcus* are internalized to the same extent (see Fig. S1 in the supplemental material). There was no significant difference in intracellular survival between three GBS strains, representing serotype III (NEM316), serotype 1a (A909), and COH1 from the hypervirulent MLST 17 clade. Thus, group B *Streptococcus* appears to persist inside macrophages for prolonged periods.

Survival is independent of serotype, MLST, or clinical presentation. To further probe strain variation in GBS, we tested macrophage survival in an extensive collection of GBS isolates (Fig. 2 and Table 1). The collection represents a range of serotypes and MLSTs and includes isolates from early-onset neonatal disease, late-onset neonatal disease, and asymptomatic colonization (19). Despite the genetic variability within this group, there was no significant difference in phagosomal survival across isolates, and all of the isolates showed significantly higher survival than that of the control organism, *L. lactis*. Thus, it appears that at 6 h postinfection, all isolates of GBS have the same ability to resist the antimicrobial actions of phagocytic cells, despite the reported high genetic diversity of the species and the strong association of different strains with different disease presentations.

Group B *Streptococcus* resides in a phagosome that recruits late endosomal markers. A mechanism by which some pathogenic microorganisms can survive phagocytic killing is to alter the maturation of the phagosome to create an environment that is

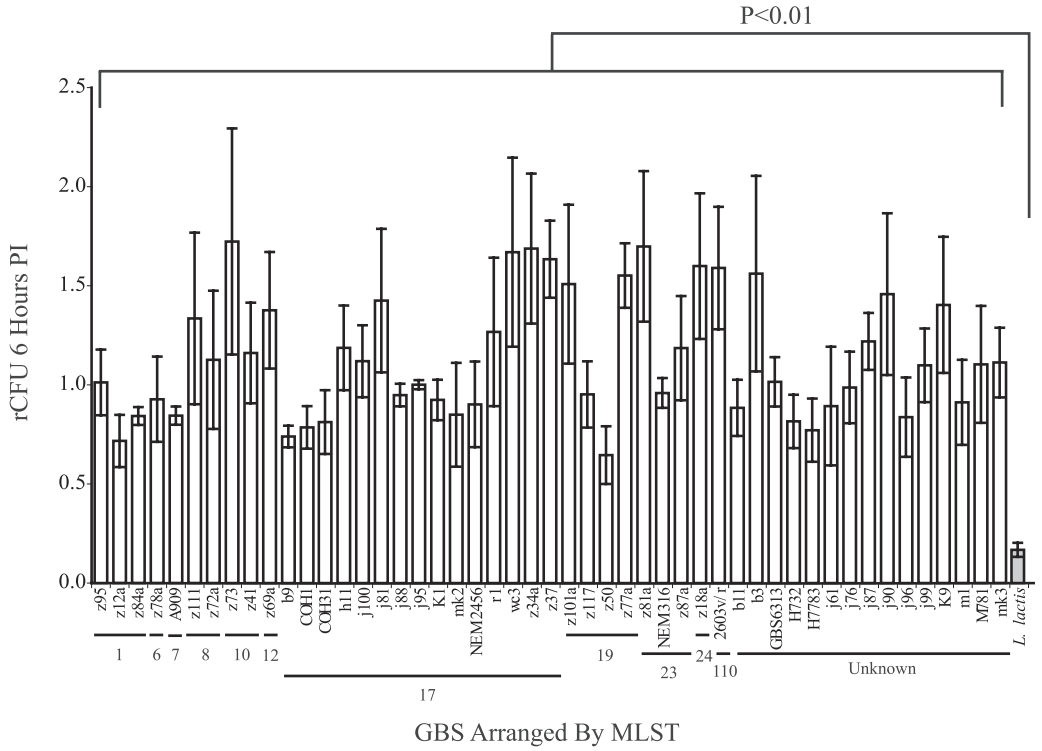


FIG 2 Intracellular survival of GBS clinical isolates in J774 macrophages. Fifty GBS isolates were assessed for intracellular survival in J774 macrophages. Survival is presented as rCFU at 6 h postinfection. GBS isolates are arranged by MLST. All GBS isolates show significantly higher survival than *L. lactis* (MG1363) (Mann-Whitney U test, $P < 0.01$, at least three repeats) but do not significantly differ from each other (Kruskal-Wallis, $P = 0.147$). Values displayed are means ± standard errors (SE) for each isolate.

more favorable for growth (15). To assess whether GBS survives within macrophages by perturbing phagosome maturation, we monitored the time-dependent acquisition of lysosome-associated membrane protein (LAMP), a glycoprotein associated with acidified lysosomal compartments (26), by GBS-containing phagosomes (Fig. 3A and B). However, the extent of LAMP acquisition by GBS-containing phagosomes did not differ from that of phagosomes containing latex beads up to 180 min postinfection (Fig. 3C). We note that, although the majority of GBS remains within an intact phagosome for the duration of the experiment, we cannot rule out the possibility that the LAMP-negative GBS may represent bacteria that have escaped from the phagosome. Taken together, however, these data indicate that the majority of GBS remains within a phagosome that acquires LAMP at a normal rate.

Response of wild-type group B *Streptococcus* to physiological stress. Within the phagosome, organisms encounter reactive oxygen species (ROS) and reactive nitrogen species (RNS), hence we reasoned that increased tolerance to these damaging molecules could explain the intracellular survival phenotype of GBS. We exposed GBS to increasing concentrations of hydrogen peroxide (H_2O_2) (Fig. 4A) *in vitro*. GBS shows an increased tolerance to hydrogen peroxide stress in comparison with the nonpathogenic organism *L. lactis*, which is highly sensitive to the chemical. However, previous data suggest that 14 mM H_2O_2 accurately reflects the ROS conditions inside an activated alveolar macrophage (6), and this concentration is sufficient to kill GBS *in vitro* (Fig. 4A). Since J774 cells are nonetheless able to support survival of the organism, the most likely explanation for this paradox is that GBS fails to induce a large oxidative burst in these host cells. In line with this, GBS-infected J774 cells did not produce significantly more ROS than uninfected macrophages (Fig. 4B), a finding which agrees with previously published data examining GBS infections in murine bone marrow macrophages and human neutrophlils (8–10). Lastly, we reduced ROS by inhibiting the NADPH oxidase of infected cells using 0.5 mM apocynin. The compound inhibited ROS production by 10% (Fig. 4B) but had no effect on the intracellular survival of wild-type GBS (Fig. 4C) or *Lactococcus* (data not shown). We note that apocynin requires activation by H_2O_2 or myloperoxidase in order to give maximal inhibition of the NADPH oxidase (45), and thus the relatively low level of inhibition we observe most likely reflects weak induction of the NADPH oxidase by intracellular GBS.

Since mature phagosomes contain reactive nitrogen species in addition to ROS, we mimicked intraphagosomal nitrosative stress by exposing GBS to increasing concentrations of sodium nitrite ($NaNO_2$), which dissociates at low pH to yield NO. In contrast to hydrogen peroxide, the tolerances of wild-type GBS and *L. lactis* to NO are similar, with neither organism showing significant lethality (data not shown). The intraphagosomal concentration of nitric oxide is around 57 μM (5); hence, this agent may be responsible for limiting intraphagosomal growth of GBS and *L. lactis* but not for killing the organisms.

Taken together, these data indicate that neither reactive oxygen nor reactive nitrogen species play a large role in control of intracellular GBS in J774 macrophages.

Acidification of the phagosome is important for GBS survival. Phagosome maturation is accompanied by a steady decrease in phagosomal pH (26). We therefore tested the sensitivity of GBS

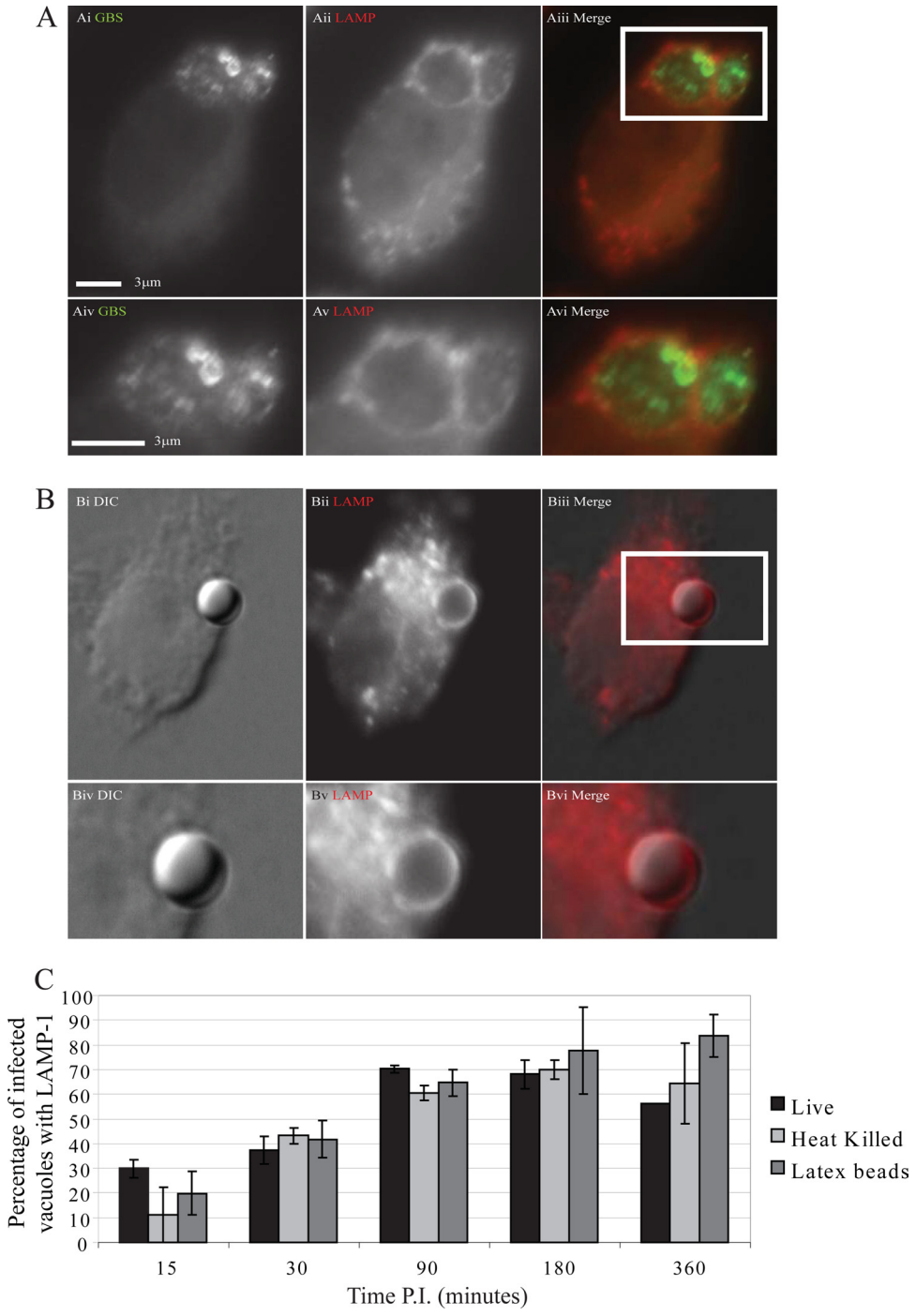


FIG 3 Group B *Streptococcus*-containing phagosomes show no delay in LAMP acquisition. J774 macrophages settled on coverslips were infected synchronously with live wild-type GBS strain NEM316, heat-killed NEM316, or latex beads. Coverslips were removed at time points postinfection, fixed, and immunostained for streptococci (green) and lysosome-associated membrane protein (red). Coverslips were visualized using a Nikon Eclipse Ti microscope with a 100× DIC objective. Images shown are from 90 min postinfection. (A) Live FITC-stained GBS (i and iv), with LAMP, stained with TRITC (ii and v), acquisition. Merged images are shown in panels iii and vi. (B) Representative image of a latex bead-containing phagosome with corresponding DIC (i and iv), LAMP (iii and iv), and merged (v and vi) images. The graph (C) shows the average percentage of GBS or bead-containing vesicles which costained for LAMP. At least 50 phagosomes were scored in each independent repeat. Peak acquisition can be seen at 90 min postinfection (P.I.), with a slight decrease at 360 min postinfection in the phagosomes containing live GBS. There is no difference observed between live organisms (black bars), heat-killed organisms (light gray), and 3-μm latex beads (dark gray) (χ^2 on raw data, $P > 0.05$), with the exception of the live organisms and latex beads at 360 min postinfection ($P = 0.004$). Error bars represent standard errors from three repeats.

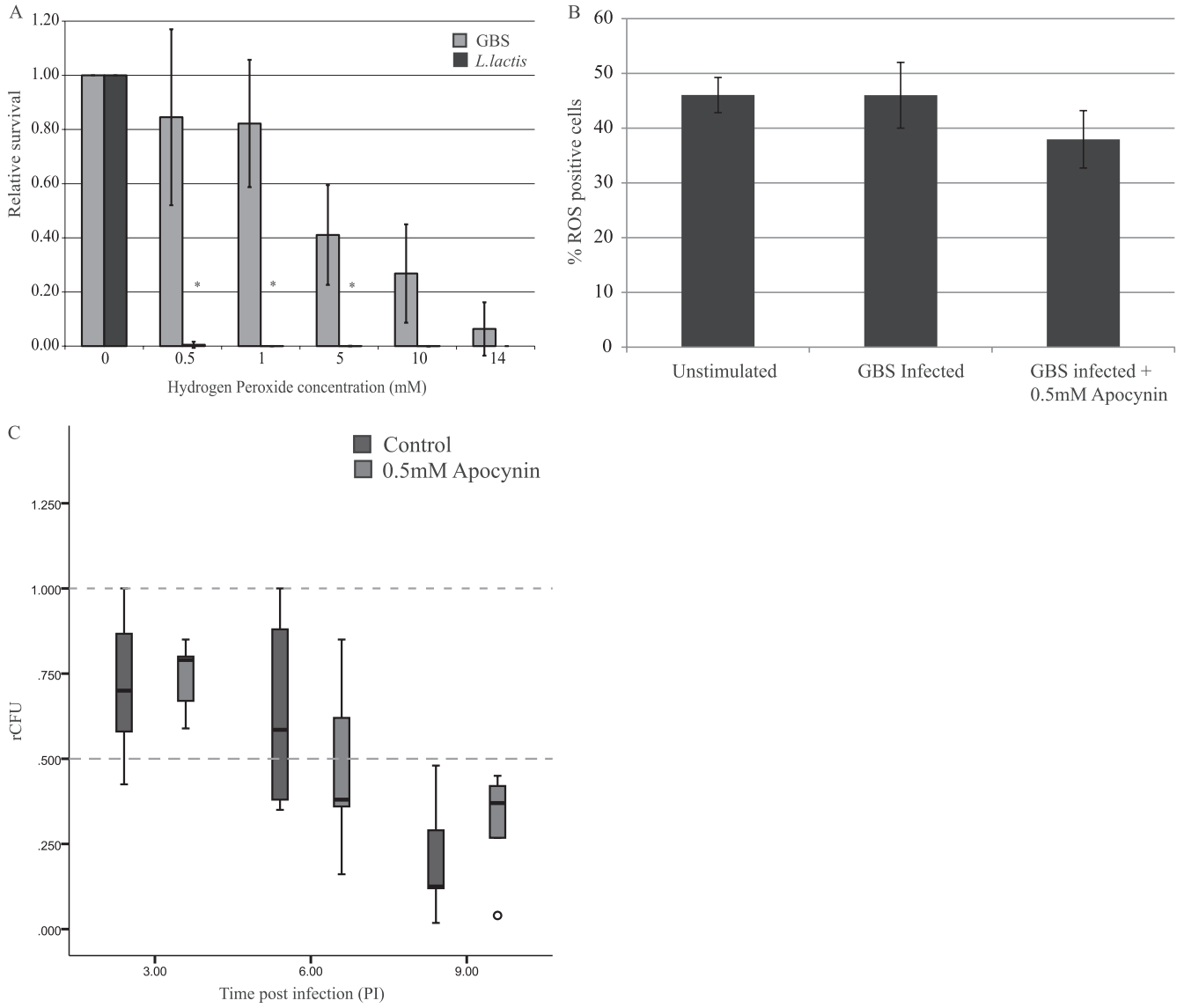


FIG 4 GBS is more tolerant of hydrogen peroxide (H_2O_2) than *L. lactis*; however, this resistance to reactive oxygen species is unlikely to be required for the intracellular persistence seen by the organism in J774 cells. (A) Survival of NEM316 (lighter bars) and *L. lactis* (darker bars) when in the presence of increasing concentrations of hydrogen peroxide. Growth is expressed as relative survival after 1 h of incubation with H_2O_2. (B) Reactive oxygen species (ROS) production was measured using flow cytometry and the dye H_2DCFDA. GBS infection does not increase ROS production, although baseline ROS levels can be inhibited by apocynin. (C) Inhibition of the reactive oxygen burst with 0.5 mM apocynin does not affect the intracellular survival of GBS (Mann-Whitney U, $P < 0.05$, $n = 5$).

to acidic conditions *in vitro*. In line with their observed intracellular survival, GBS strains were fully tolerant of conditions down to pH 4.4, with loss of viability occurring only at pH 3.6. Acidification of the phagosome can be blocked by concanamycin A (ConA), an inhibitor of the vacuolar ATPase (vATPase) phagosome membrane proton pump that is responsible for the influx of protons leading to the decreasing pH (13). We therefore examined the effect of blocking phagosomal acidification on GBS intracellular survival. After infecting J774 macrophages with GBS strain NEM316 or the control organism *L. lactis*, we inhibited acidification of the macrophage phagosome using 0.1 μM concanamycin A (a concentration that has no effect on J774 survival or the survival of GBS in Todd Hewitt broth) (data not shown). Concanamycin A treatment significantly reduced the survival of wild-type GBS, but not *L. lactis*, at 6 h postinfection (Fig. 5A). This suggests that GBS not only resides in a mature phagosome but also requires the normal phagosome acidification and subsequent maturation to survive within this niche.

The increased pH of the phagosome following concanamycin A treatment will prevent the dissociation of iron from transferrin, which does not occur at a pH below 7, and we therefore considered the possibility that the reduced survival of GBS under these conditions may be an indirect result of iron deprivation. However, addition of FeNTA (a nonphysiological iron chelator that releases iron in a pH-independent manner over a range of pH 5 to 8 used in previous similar studies [2, 30]) did not alter the intracellular survival phenotype of GBS either in the presence or absence of ConA (Fig. 5B). Thus, iron availability appears to have limited

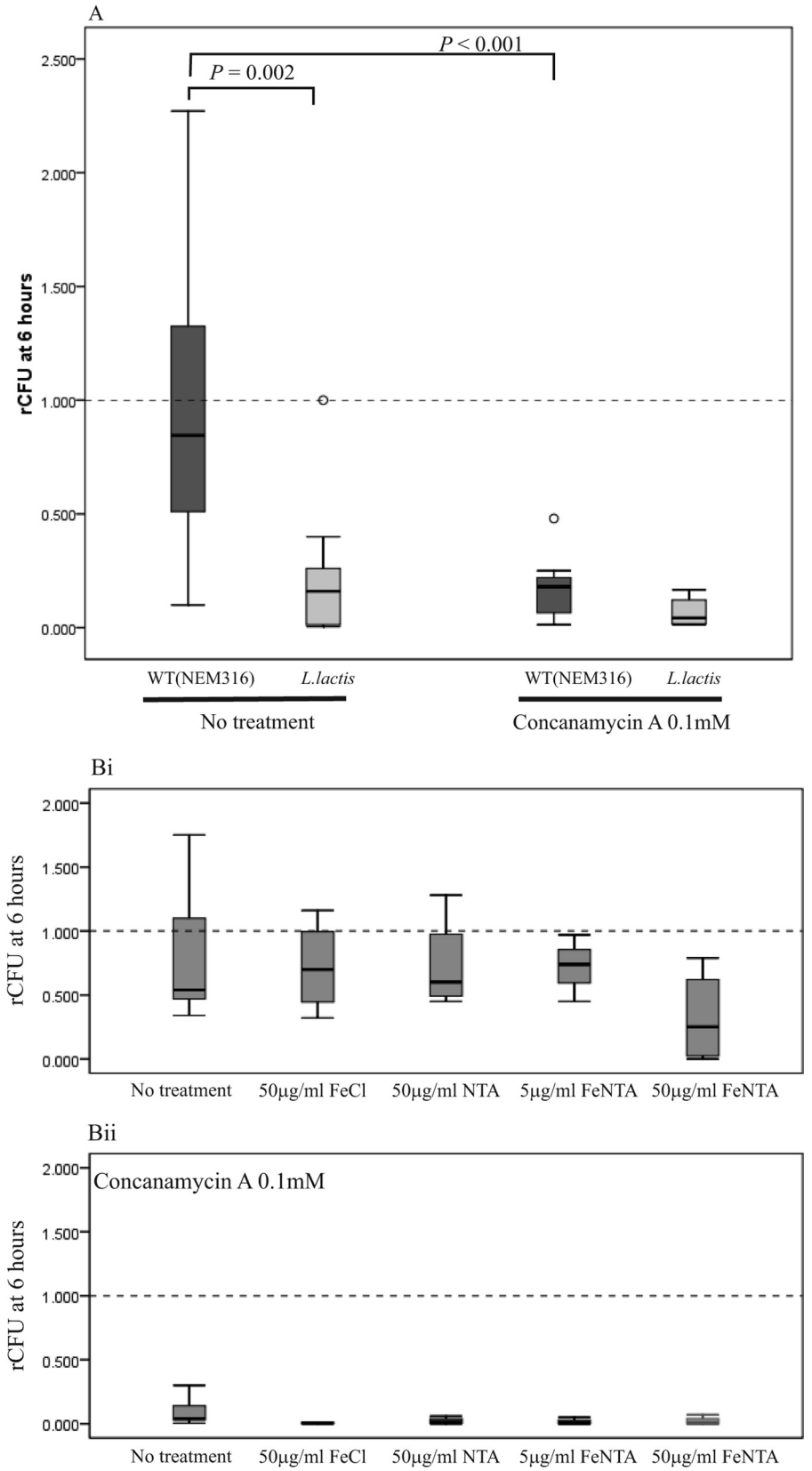
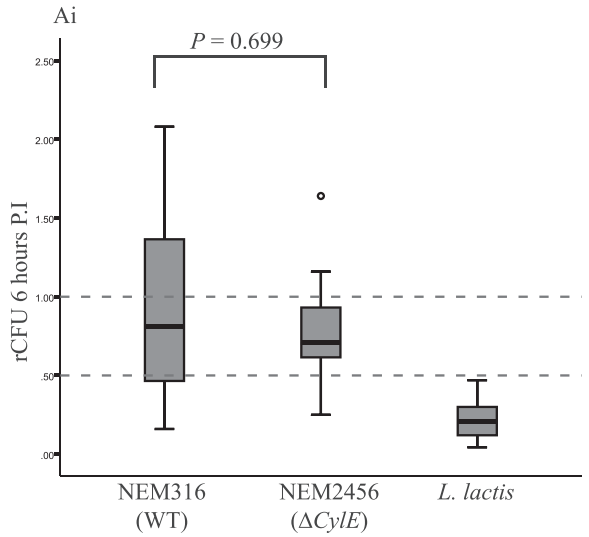
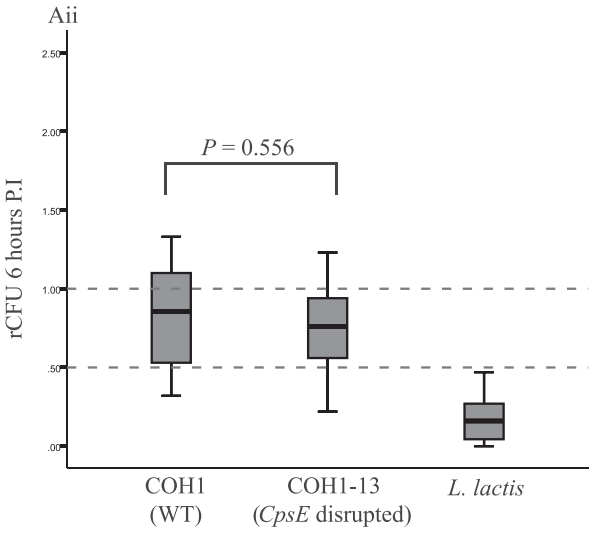
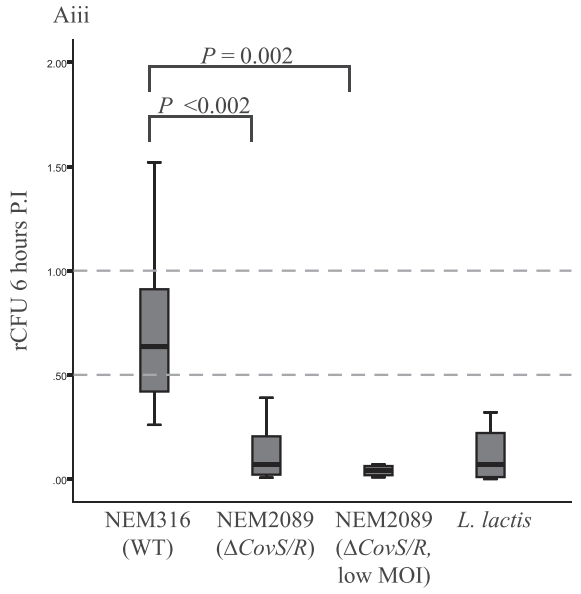
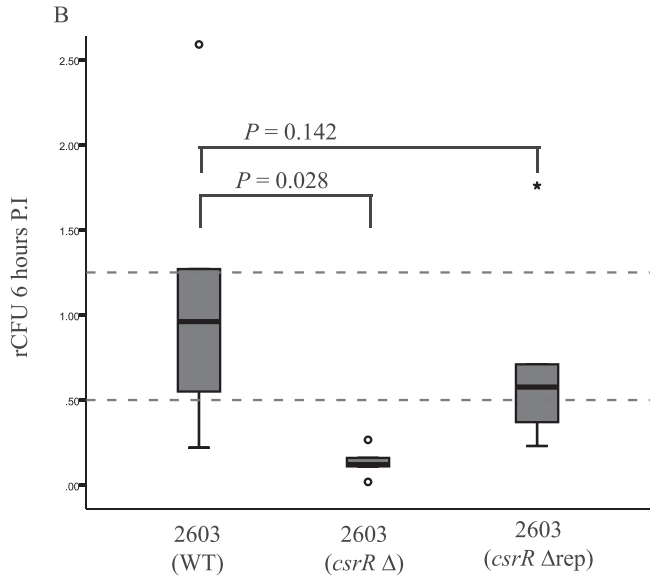


FIG 5 Blocking acidification of the phagosome reduces the intracellular survival of GBS, independent of iron availability. (A) J774 macrophages infected with NEM316 and *L. lactis* were treated with the vATPase inhibitor concanamycin A (ConA). Under these conditions, the number of viable intracellular streptococci isolated at 6 h postinfection is significantly reduced (Mann-Whitney U, $P < 0.001$, $n \geq 5$). (B) J774 macrophages infected with strain NEM316 incubated in the presence (Bii) or absence (B) of 0.1 mM concanamycin A. The graphs show rCFU at 6 h postinfection, demonstrating the effect of the addition of FeCl, NTA, and FeNTA to ConA-treated and nontreated cells. The nonphysiological iron chelator complex FeNTA failed to recover the concanmycin A-associated loss of viable intracellular streptococci. There is no significant difference between the no-treatment control and the infected cells that received iron supplementation in either ConA-treated ($P = 0.572$) or untreated ($P = 0.428$) cells (Kruskal-Wallis test, $n = 6$).


Ai
P = 0.699
rCFU 6 hours P.I
2.50
2.00
1.50
1.00
.50
.00
NEM316 (WT)
NEM2456 (ΔCylE)
L. lactis


Aii
P = 0.556
rCFU 6 hours P.I
2.50
2.00
1.50
1.00
.50
.00
COH1 (WT)
COH1-13 (CpsE disrupted)
L. lactis


Aiii
P = 0.002
P <0.002
rCFU 6 hours P.I
2.00
1.50
1.00
.50
.00
NEM316 (WT)
NEM2089 (ΔCovS/R)
NEM2089 (ΔCovS/R, low MOI)
L. lactis


B
P = 0.142
P = 0.028
*
rCFU 6 hours P.I
2.50
2.00
1.50
1.00
.50
.00
2603 (WT)
2603 (csrR Δ)
2603 (csrR Δrep)

impact on GBS survival, which likely reflects the relatively short time period during which the organism resides within the host cell.

Phagosomal survival is dependent on the presence of the CovS/R regulatory system but not the capsule or β-hemolysin/cytolysin and associated pigment. Previous work has highlighted a number of virulence factors required by GBS for full virulence in rodent models of infection (16, 22, 28, 31) (for a review, see reference 32). We asked whether any of these factors were required for intracellular survival in macrophages by infecting J774 cells with GBS mutants with deletions of well-characterized virulence determinants: the polysaccharide capsule (*cpsE*), the β-hemolysin/cytolysin (*cylE*), and the two-component regulatory system (TCS) *covS/covR*.

J774 macrophages were infected with GBS strains as previously described, and after 6 h the cells were lysed and the number of viable GBS CFU were counted. A GBS strain lacking the hemolysin/cytolysin gene *cylE*, which also prevents production of the caro tenoid pigment (NEM2456), did not show any significant difference in intracellular survival relative to isogenic wild-type strains (Fig. 6Ai). Similarly, an acapsular mutant (COH1-13) also exhibited continued intracellular survival (Fig. 6Aii). Neither of these mutants showed any difference in uptake into macrophages compared to that of the parental strains (see Fig. S1 in the supplemental material), and this trend in survival extended to at least 9 h postinfection (see Fig. S2 in the supplemental material).

The capsule has been reported as a major virulence factor since it exerts antiphagocytic properties, such as preventing complement deposition (35). In our serum and opsonin-free conditions, however, the presence of a capsule does not dramatically affect uptake. Once internalized, the GBS capsule does not play a large role in survival of the organism, in line with previously described data (12). In marked contrast, however, strain NEM2089, which has the *covS/covR* genes deleted, showed dramatically impaired intracellular survival and was no longer distinguishable from the nonpathogenic control organism (Fig. 6Aiii). To confirm the role of the CovS/CovR two-component system, we repeated the assay using a strain deleted in only the regulator element CsrR (CovR) in a different strain background, 2603 (Fig. 6B). This strain showed a similar significant decrease in intracellular survival compared to that of the isogenic parent and the repaired strain, confirming the importance of the global regulator across the species.

In order to try to explain the reduced intracellular survival of the CovS/CovR deletion strain, we tested the susceptibility of this mutant to a range of phagosomal stress factors. However, the CovS/R mutant was indistinguishable from wild-type GBS in its tolerance to low pH and oxidative and nitrosative stress, and intracellular survival was also unchanged in cells treated with the NADPH oxidase inhibitor apocynin (data not shown). Since the production of reactive oxygen and nitrogen species is thought to be an early phagosome response to ingestion of microorganisms, the tolerance of these TCS mutants to such stresses reflects their ability to survive to the same extent as the wild type at early time points and only later succumb to host killing (see Fig. S2 in the supplemental material).

Since the CovS/R mutant is phagocytosed more efficiently than wild-type GBS, (see Fig. S1 in the supplemental material), probably because it is hyperadherent (28), we considered the possibility that the CovS/R survival defect may reflect overloading of host macrophages. However, reducing the number of internalized bacteria at time zero to the same level as that of the wild-type organism does not change the survival kinetics (see Fig. S1 in the supplemental material and Fig. 6iii). Thus, the survival defect of the CovS/R mutant occurs independently of bacterial burden and reflects a genuine defect in intracellular survival, which may explain the importance of this TCS in rodent models of virulence (22, 28).

DISCUSSION

Although group B streptococci are not thought to be intracellular pathogens *per se*, we and others have shown that viable organisms can be recovered from phagocytic cells for longer than nonpathogenic organisms (12, 49). In clinical GBS disease, the organism achieves a high level of bacteremia, which precedes life-threatening septicemia and meningitis. Intracellular survival within phagocytes may contribute to this disease progression by reducing the effectiveness of phagocyte clearance. In addition, some pathogens actively exploit phagocytes to facilitate traffic between host tissues, and it is possible that a related process may underlie GBS dissemination (37, 48).

In this study, we sought to further understand the interaction between GBS and host macrophages. GBS shows considerable interspecies variability, with different serotypes associated with differences in disease presentation (4, 51). By extensively testing a diverse collection of GBS isolates (19), we have now shown that there are no significant differences between strains, serotypes, or MLST groups in their capacity to survive within macrophages. These data suggest that epidemiological variation in disease progression and hypervirulence may result from differences in colonization or extracellular growth rather than differences in the interaction with phagocytes. Interestingly, such a conclusion is supported by recently published data indicating that one explanation for the increased virulence of ST-17 clones of GBS is the observation that they overexpress the cell wall surface protein HgvA, leading to increased adherence to host epithelia (46).

The phagosome of macrophages is an inhospitable environment for any ingested microorganisms, being both highly acidic and high in reactive oxygen and nitrogen species. GBS is known to have several mechanisms that protect against oxidative stress, such as the production of superoxide dismutase (SodA) and free-radical scavenging carotenoid pigment associated with the *cylE* gene (31, 39). In our assay, we find that *cylE* is dispensable for intracellular survival, in contrast to previous work which suggests

FIG 6 The two-component regulatory system CovS/CovR, but neither the capsule nor the *cylE* operon, is required for the intracellular survival of GBS in J774 cells. Intracellular survival in J774 cells 6 h postinfection (P.I.) was assessed as described previously. NEM2456 (Ai), a strain deficient in the *cylE* gene, and COH1-13 (Aii), a strain disrupted in the capsule gene *cpsE*, were compared to the isogenic parent strains NEM316 and COH1, respectively. In both cases, survival was not significantly different between the mutant and the parent. (Aiii) NEM2089, a derivative of NEM316 that is deleted in *covS/covR*, shows a significantly reduced ability to survive inside J774 macrophages. This cannot be explained by a higher level of internalization of this mutant, since reducing the MOI by 10-fold (CovS/R low MOI) does not alter the intracellular survival rate. To confirm the importance of the two-component system in survival, we repeated the assay using a strain deleted in the CsrR (CovR) gene in a different parental background (B). This strain shows a similar defect in survival, which is restored in the complemented strain. (Mann-Whitney U test, $n > 4$).

that the associated carotenoid pigment is required for intracellular survival due to its ability to scavenge free radicals (31). We have shown that GBS is sensitive *in vitro* to levels of hydrogen peroxide that are thought to be physiologically relevant in activated phagocytic cells (6); however, the inhibition of ROS through the NADPH oxidase does not affect intracellular survival of wild-type GBS. This would support our findings and previous work suggesting that GBS-containing phagosomes fail to undergo a full oxidative burst when the organism has been ingested in the absence of opsonizing antibody (10, 14).

One method used by microorganisms to avoid being killed in the phagosome is to actively perturb phagosome maturation. For instance, in both *Burkholderia cenocepacia* and *Mycobacterium tuberculosis*, a delay in phagosome maturation is associated with intracellular survival (27, 38). In contrast, we have shown that a marker of phagosome maturation, lysosome-associated membrane protein (LAMP), is recruited normally to the GBS-containing phagosome membrane, although we cannot currently rule out the possibility that other steps in phagosome maturation may be altered. However, normal acidification of the phagosome appears essential for GBS survival, since inhibiting the vATPase with concanamycin A reduces the survival of the organism. One potential explanation for this finding is that concanamycin A, in changing the phagosomal pH, prevents transferrin from releasing iron within this organelle, such that GBS is now residing in an iron-limited environment. The lack of available iron in the neutral phagosome has been shown to be the limiting factor in survival of *Francisella tularensis* and *Legionella pneumophilia* within cells that have been treated with drugs that prevent acidification (7, 17). However, the fact that iron supplementation did not rescue intracellular survival, combined with previous data showing that GBS can grow in low-iron conditions due to the presence of the siderophore receptor FhuD and other less well-established mechanisms (11), suggests that iron limitation is unlikely to explain the reduced survival of GBS in ConA-treated phagocytes. Instead, it may be that GBS requires a low pH phagosome environment for intracellular survival because of pH-dependent transcriptional responses, such as those that occur during intracellular survival of the fungal pathogen *Cryptococcus neoformans* (30). In this context, it is intriguing that the CovS/CovR pH-responsive regulator is required for intracellular survival.

What mechanisms may GBS use to survive the hostile environment of the phagolysosome? Our data demonstrate that loss of CovS/CovR abrogates intracellular survival, in line with previous data that has shown this mutant to be altered in virulence in a rodent model (22, 28, 29). The CovS/CovR system (named Cov from *Streptococcus pyogenes* control of virulence, sensor and regulator system but also known as CsrR/S or capsule synthesis regulator) is known to be an important transcriptional regulator in pathogenic streptococci, regulating approximately 7% of the GBS genome, particularly during adaptation to host conditions (23, 28).

GBS is clearly well adapted to growth under conditions of low pH, since it can ferment carbohydrates to lactic acid and, as a commensal, will reside in the vagina at a pH of 3.8 to 4.5. However, during a systemic infection, the organism must be able to respond to the much higher pH of blood (7.4). Microarray analysis showed that expression of 18% of the GBS genome is altered in response to acid shock, and the majority of these changes are controlled by the CovS/CovR operon (44). Since the phagosome steadily decreases in pH from 6.1 in the early phagosome to 4.5 in the phagolysosome, it is likely that the sensor CovS will be activated during phagosome maturation. Our finding that GBS survival is impaired either by the loss of CovS/CovR or by raising phagosomal pH could therefore be explained by a model in which CovS/CovR senses the acidified nature of the phagosome to trigger stress responses that allow phagosomal survival. Without that trigger (either by a lack of acidification during concanamycin A treatment or by the loss of the sensor system in the CovS/CovR mutant), GBS is unable to tolerate the antimicrobial environment of the phagosome. Interestingly, the CovS/CovR two-component system (TCS) and corresponding regulon do not appear to play a role in the organism's sensitivity to oxidative or nitrosative stress nor its ability to tolerate acid.

The main gene families that CovS/CovR has been shown to upregulate in response to acid are those involved in metabolic transport systems, including the iron siderophore receptor FhuD and the genes required for polyamine synthesis (44). This suggests that in acidic conditions, GBS enters a highly metabolic stage, and the low pH of the phagosome could be a favorable environment to trigger this. Without the pH-regulated metabolic change, the organism may not be able to remain viable in the nutrient-limiting phagosome. In addition, GBS undergoes changes in the cell wall and capsule composition in response to acid that may be relevant to intracellular survival. In particular, the pilus protein, which provides resistance to antimicrobial peptides, is upregulated (33), while the cell wall protein BibA, which has been shown to increase opsonophagocytic killing (43), is downregulated at low pH (31, 33, 39, 44). Strains of GBS with CovR deleted have been shown to stimulate an increased proinflammatory cytokine response in host cells compared to parental strains. This suggests that within the CovR/CovS regulons, there is control of expression of genes which produce host-modulating factors (29). Therefore, it should also be considered that the reduced survival of the deletion mutant in macrophages could be attributed to the macrophages being more activated in the absence of CovS/CovR-dependent responses.

In conclusion, our data support a model in which all strains of GBS share an ability to survive in phagocytic cells, via a pH-dependent transcriptional response that occurs within the phagosome. Such an ability likely provides a virulence capacity to GBS, which is then augmented by the presence of strain-dependent virulence traits, such as the expression of HgvA.

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