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Loss of SigB in *Listeria monocytogenes* Strains EGD-e and 10403S Confers Hyperresistance to Hydrogen Peroxide in Stationary Phase under Aerobic Conditions

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ABSTRACT

SigB is the main stress gene regulator in *Listeria monocytogenes* affecting the expression of more than 150 genes and thus contributing to multiple-stress resistance. Despite its clear role in most stresses, its role in oxidative stress is uncertain, as results accompanying the loss of *sigB* range from hyperresistance to hypersensitivity. Previously, these differences have been attributed to strain variation. In this study, we show conclusively that unlike for all other stresses, loss of *sigB* results in hyperresistance to H_2O_2 (more than 8 log CFU ml^{-1} compared to the wild type) in aerobically grown stationary-phase cultures of *L. monocytogenes* strains 10403S and EGD-e. Furthermore, growth at 30°C resulted in higher resistance to oxidative stress than that at 37°C. Oxidative stress resistance seemed to be higher with higher levels of oxygen. Under anaerobic conditions, the loss of SigB in 10403S did not affect survival against H_2O_2, while in EGD-e, it resulted in a sensitive phenotype. During exponential phase, minor differences occurred, and this result was expected due to the absence of *sigB* transcription. Catalase tests were performed under all conditions, and stronger catalase results corresponded well with a higher survival rate, underpinning the important role of catalase in this phenotype. Furthermore, we assessed the catalase activity in protein lysates, which corresponded with the catalase tests and survival. In addition, reverse transcription-PCR (RT-PCR) showed no differences in transcription between the wild type and the Δ*sigB* mutant in various oxidative stress genes. Further investigation of the molecular mechanism behind this phenotype and its possible consequences for the overall phenotype of *L. monocytogenes* are under way.

IMPORTANCE

SigB is the most important stress gene regulator in *L. monocytogenes* and other Gram-positive bacteria. Its increased expression during stationary phase results in resistance to multiple stresses. However, despite its important role in general stress resistance, its expression is detrimental for the cell in the presence of oxidative stress, as it promotes hypersensitivity against hydrogen peroxide. This peculiar phenotype is an important element of the physiology of *L. monocytogenes*, and it might help us explain the behavior of this organism in environments where oxidative stress is present.

Listeria monocytogenes is a Gram-positive bacterium that causes listeriosis, a serious and potentially lethal foodborne illness (1). Despite its low incidence, listeriosis has a high mortality rate (30%), making it the most deadly foodborne disease in the United Kingdom and the United States, as it claims more lives than any other foodborne pathogen (1, 2). One of the key attributes that makes *L. monocytogenes* such a successful pathogen is its ability to survive and persist in a wide range of harsh environments both outside and within the human host (3). One of the most important stresses *L. monocytogenes* has to withstand, in order to survive and cause disease, is oxidative stress. Oxidative stress can occur in the environment where metal or nonmetal redox catalysts are present, during disinfection with oxidative disinfectants, and during processing of foods with ozone or plasma. Furthermore, during the intracellular stage of *L. monocytogenes* infection, the bacterium encounters oxidative stress within the phagolysosome during phagocytosis.

The alternative sigma factor σ^B plays an important role in the stress responses of several Gram-positive bacteria (4). In *L. monocytogenes*, SigB regulates the expression of more than 150 genes (5), contributing to resistance to multiple stresses, including oxidative stress, acid, heat, salt, and bile acids (6–9). Recent evidence shows that the induction of SigB in *L. monocytogenes* occurs in the early exponential phase of growth, eventually reaching maximum levels at early stationary phase (10).

Highlighting its role in stress resistance, the deletion of *sigB* in *L. monocytogenes* leads to sensitivity against various stresses, which, in most cases, can be explained at the molecular level (6–9). An exception to this has been observed with oxidative stress in *Bacillus cereus* (11), where it has been reported that the Δ*sigB* mutant is more resistant than the wild type (WT). In the case of *L. monocytogenes*, there is no consensus about the role of SigB in oxidative stress, with various studies showing that the deletion of

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sigB leads to sensitivity to oxidative stress (7, 9), while others report increased resistance (12). This discrepancy has previously been attributed to strain variability (12). In this study, we investigate possible reasons for previous discrepancies and the role of SigB in oxidative stress under various environmental conditions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. WT *L. monocytogenes* strains 10403S and EGD-e (both belong to serotype 1/2a) and their isogenic Δ*sigB* mutants were used throughout this study. EGD-e, 10403S, and Δ*sigB* mutants were all constructed during previous works (13, 14) and have been used extensively in work on the role of SigB in *L. monocytogenes* (7, 11). Stock cultures were stored at −80°C in 7% (vol/vol) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Dorset, United Kingdom). Prior to experiments, stock cultures were streaked onto brain heart infusion (BHI) agar (LAB M, Lancashire, United Kingdom) and incubated at 37°C or 30°C overnight. A single colony from this medium was transferred to 3 ml of sterile BHI broth (LAB M) and incubated overnight at 37°C or 30°C with shaking (160 rpm). Subsequently, a portion of these overnight cultures served as the inoculum (1% [vol/vol]) to prepare the cultures that were used in the experiments. These cultures were prepared in 250-ml conical flasks containing 20 ml of the same medium as the one used for the inoculum and incubated overnight at 37°C or 30°C with shaking (130 rpm). For anaerobic growth, cultures were grown in 20 ml of BHI in Sterilin Quickstart Universal polystyrene containers (Thermo Scientific, Loughborough, United Kingdom) and placed in an anaerobic cabinet at 37°C with a gas atmosphere maintained at a 80:10:10 N_2:CO_2:H_2 ratio.

Survival in the presence of hydrogen peroxide. Stationary-phase cells were grown for approximately 18 h, while mid-exponential-phase cells were grown for 4 to 5 h until an optical density at 600 nm (OD_{600}) of ~0.75 was reached on a spectrophotometer (Spectronic 200; Thermo Fisher Scientific, Loughborough, United Kingdom) and subsequently were challenged with H_2O_2 (Sigma-Aldrich, Gillingham, United Kingdom). To allow comparisons, the survival of anaerobically grown cells had to be performed at a point of growth similar to that of the aerobically grown cells. Therefore, prior to the experiments, OD_{600} measurements were taken for 24 h to construct aerobic and anaerobic growth curves (data not shown). In these experiments, it was determined that the 21-h point of the anaerobic growth curves corresponded to the 18-h point in the aerobic ones. Subsequently, cells grown anaerobically were challenged at stationary phase following ~21 h of growth.

A 30% solution of H_2O_2 (Sigma-Aldrich, Dorset, United Kingdom) was added to the flasks at various concentrations for each condition and type of cells, but only one representative set of results is presented here. Depending on the H_2O_2 resistance of each strain, final concentrations of 0.4%, 0.43%, 1%, 3%, and 4.5% (vol/vol) are presented here, and samples were taken at 0 min and every 20 min. Prior to assessment of H_2O_2 survival, preliminary experiments were performed to define the concentrations of H_2O_2 that should be used with each of the strains and conditions to avoid rapid death or complete survival that would allow comparison between the *sigB* mutants and their corresponding WT strains. During the experiments, cultures were kept at the temperature at which they were grown overnight (30°C and 37°C). Prior to and after the addition of H_2O_2, samples were taken, and serial dilutions were prepared in maximum recovery diluent (MRD; Oxoid, United Kingdom) and spread on BHI agar plates that were incubated at 37°C for 2 days. Subsequently, CFU were enumerated to assess the concentration of cells in the cultures at each time point (every 20 min). All experiments were performed at least in triplicate, and the average and standard deviation were calculated.

Disk diffusion assay. Cells of the EGD-e WT, 10403S WT, and their corresponding Δ*sigB* mutants were grown overnight in Mueller-Hinton broth at 30°C or 37°C with shaking (160 rpm). Subsequently, overnight cultures were diluted to an OD_{600} of 0.2, and 100 μl was spread onto Mueller-Hinton agar (MHA; Oxoid, Basingstoke, United Kingdom). Then, 10 μl of 30% (vol/vol) H_2O_2 was pipetted onto Whatman 3MM paper disks (0.7-cm diameter), and these disks were placed on top of the agar and incubated for 18 h at the same temperature as the overnight culture (30°C or 37°C). The zones of inhibition (in millimeters) were taken as a measure of H_2O_2 sensitivity. The zones of inhibition were measured in three dimensions, and the mean values and standard deviations were calculated. All experiments were performed on six independent biological replicates, and statistical analysis was performed as described below.

Catalase test. Overnight cultures were grown as described above, and subsequently, 5 ml was transferred into Sterilin Quickstart Universal polystyrene containers and 100 μl of 30% H_2O_2 was added to the aerobic and anaerobic cultures, respectively. The oxygen released in the form of bubbles was visually monitored and photographed after a 5-min period as an indication of catalase activity.

Catalase activity in protein extracts. Measurement of the catalase activity in protein extracts was performed as described previously (15), with modifications to assess the intracellular activity of this enzyme. In short, following the removal of the growth medium, proteins were extracted using a sonication-based method, as described previously (16), using 20 ml stationary-phase cultures grown for 18 h. The concentrations of protein extracts were determined by the *RC DC* protein assay kit (Bio-Rad). Extracts were normalized to 0.5 mg ml^{-1}, and 25 μl of each was added to 1 ml of an aqueous solution of 1% H_2O_2 contained in a quartz cuvette already placed in a UV spectrophotometer set up to record absorbance at 240 nm (λ at which H_2O_2 absorbs). Subsequently, measurements were taken every 10 s, and the reduction in the intensity of signal represented the H_2O_2 degradation due to catalase. The above-mentioned concentrations of protein and H_2O_2 used were defined in preliminary experiments, which aimed to avoid any rapid formation of bubbles in the cuvette causing erratic changes in the absorbance that would make any measurement impossible.

DO measurements. The concentration of dissolved oxygen (DO) present in bacterial cultures was assessed using an optical sensor (InLab OptiOx) attached to a SevenExcellence S900 benchtop instrument (Mettler Toledo, Columbus, OH, USA). The sensor was calibrated daily using a two-point calibration. The instrument was blanked using tablets, and atmospheric saturation was achieved by placing the sensor in the air. Triplicate measurements of DO were taken from both aerobic and anaerobic cultures grown under the same conditions described for the survival assays.

Transcriptional analysis of genes contributing to oxidative stress. Transcriptional analysis was performed in the 10403S WT and the corresponding *sigB* mutant. The transcription of genes responsible for resistance against oxidative stress was quantified as previously described by Karatzas et al. (17) following real-time reverse transcription-PCR (RT-PCR). The efficiencies of the primer pairs (shown in Table 1) were all close to 2, and these values were used for efficiency correction in the quantification step. In all cases, aerobic cultures were grown for ~18 h in BHI, and samples were taken for RNA isolation. RNA was isolated with the use of the RNeasy midi kit (Qiagen, Manchester, United Kingdom). RNA quality was assessed with the use of a 2100 Bioanalyzer (Agilent, Cheshire, United Kingdom), and in all samples used, the RNA integrity number (RIN) was between 8 and 10. Subsequently, RNA was converted to cDNA with the use of random primers and the SuperScript III reverse transcriptase kit (Invitrogen, Thermo Fisher Scientific, Paisley, United Kingdom). Relative expression was calculated as the ratio between the expression of each of the target genes (*kat*, *lmo0367*, *lmo1604*, and *tpx*) and the expression of the 16S rRNA gene, which served as the reference gene in each cDNA sample. Calculations were carried out according to the advanced relative quantification settings of the LightCycler 480 software program, with PCR efficiency correction performed as described previously (17). The relative expression of each gene was calculated by a comparison of its expression relative to that of the 16S rRNA gene.

Caco-2 proliferation assays. The gentamicin protection assay was performed with the strains, as described previously (5), with minor mod-

TABLE 1 Primers used in this study

Primer[a]	Sequence (5′ to 3′)	Efficiency
katF	CGCACGGGAAATTTGTTACT	2.05
katR	GGTCAGGCTTCAAGGAATGA	
lmo0367F	TCATGTGCGCCTAGCCAAAG	2.10
lmo0367R	CCAATTGGGACGGTGTATTC	
lmo1604F	AGGCACACAAGCTCCAAGAT	1.93
lmo1604R	CGCAGCAAGAGGGTAGTTTA	
tpxF	GCGTTGTTCCTTCCATTGAT	1.96
tpxR	ATCGCGGTGGTCAGATAAAG	
16SF	TGGGGAGCAAACAGGATTAG	2.27
16SR	TAAGGTTCTTCGCGTTGCTT	

[a] Primers were designed based on the *L. monocytogenes* EGD-e published sequences from the ListiList website (http://genolist.pasteur.fr/ListiList/) except katF, katR, 16SF, and 16SR, which were designed based on the *Listeria monocytogenes* 10403S cont5.3 whole-genome shotgun sequence (http://www.ncbi.nlm.nih.gov/genome/159?genome_assembly_id=159669). All primers designed on EGD-e background were confirmed to contain no mismatches with the 10403S background using Standard Nucleotide Blast.

ifications. Two days before the invasion assays were performed, 1.5×10^5 Caco-2 human colon adenocarcinoma cells (European Collection of Cell Cultures no. 86010202) were seeded in 24-well plates in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 1% (wt/vol) nonessential amino acids, and 20% (vol/vol) fetal bovine serum supplemented with 100 U ml^{-1} penicillin-streptomycin (Sigma). Thirty minutes before coincubation, the medium in each well was replaced with prewarmed fresh medium without antibiotics. The OD_{600} values of stationary-phase bacterial cultures grown in BHI broth overnight at 37°C were determined, all cultures were washed twice with sterile phosphate-buffered saline (PBS), and the concentrations were adjusted to obtain similar OD_{600} values. We previously confirmed that there was a good correlation between OD_{600} and the number of cells for 10403S, as assessed by comparing the numbers of CFU and OD_{600} values. Coincubation was performed with approximately 2×10^7 CFU (multiplicity of infection [MOI], 50) of stationary-phase bacteria of the 10403S strain for 45 min at 37°C. Subsequently, Caco-2 cells were washed twice with PBS and suspended in Dulbecco's modified Eagle's medium containing 150 mg $liter^{-1}$ gentamicin. After 45 min of incubation at 37°C, cells were washed twice with sterile PBS and lysed with 2 ml of Triton X-100 (1% [vol/vol]) in PBS. Following incubation for 5 min at 37°C, cell lysates were serially diluted and spread on BHI agar to determine the number of intracellular bacteria. Subsequently, to assess intracellular proliferation, intracellular bacteria were determined in a similar way every 2 h until 12 h postinvasion. This experiment was repeated 8 times, and for each time point, statistical analysis was performed using a paired *t* test. *P* values of >0.05 were considered statistically significant.

Statistical analysis. In all cases, experiments were run at least in triplicate (unless stated), and the results were assessed with a paired Student *t* test. At a *P* value of <0.05, results were deemed statistically significant.

RESULTS

Survival in the presence of hydrogen peroxide. In both 10403S and EGD-e strains, the Δ*sigB* mutant was significantly more resistant to H_2O_2 than the WT at stationary phase when cultured under aerobic conditions ($P < 0.05$). When these cells of WT 10403S were challenged with 3% H_2O_2 at 30°C (Fig. 1A) or 37°C (Fig. 1B), they showed between 7- and 8-log reduction, while the numbers of the Δ*sigB* mutant remained almost unaffected (less than 1-log reduction). Similarly, the EGD-e Δ*sigB* mutant grown at 30°C (Fig. 1C) or 37°C (Fig. 1D) was not affected by 3% H_2O_2, demonstrating major resistance against H_2O_2. Notably, the EGD-e WT was not affected at 30°C (Fig. 1C), but it showed a >8-log reduction in CFU at 37°C when exposed to 3% H_2O_2 (Fig. 1D). To obtain a measurable effect for EGD-e at 30°C, we increased the concentration of H_2O_2 from 3% to 4.5%. Under these conditions, a reduction of more than 5 logs was achieved for the WT, while the *sigB* mutant remained unaffected (Fig. 2). From these data, we concluded that WT cells of both strains grown at 30°C were more resistant than those grown at 37°C. Furthermore, EGD-e seemed to be more resistant to H_2O_2 stress than 10403S.

Despite the hyperresistance of the *sigB* mutant compared to the WT under aerobic conditions at stationary phase, the results obtained under anaerobic conditions were different. When cells of EGD-e grown anaerobically to stationary phase at 37°C were challenged with 0.4% H_2O_2, no reduction in CFU occurred in both the WT and its isogenic Δ*sigB* mutant within 60 min, while when they were challenged with 3% H_2O_2, CFU were reduced to below the detection limit within the first 20 min (data not shown). However, when the same cells were challenged with 1% H_2O_2, the WT was significantly more resistant than the Δ*sigB* mutant ($P < 0.05$; Fig. 3A). There was a statistically significant ($P < 0.05$) reduction of at least 6 logs for the EGD-e Δ*sigB* mutant within 20 min, while such a reduction occurred in the WT only after 60 min. However, under anaerobic conditions and in stationary phase for 10403S, there seemed to be no statistically significant difference ($P > 0.05$) between the WT and Δ*sigB* mutant at 0.4% H_2O_2 (Fig. 3B), while when 1% and 3% H_2O_2 were used, the numbers of both WT and the Δ*sigB* mutant were reduced to below the detection limit within the first 20 min (data not shown). Under anaerobic conditions, EGD-e was also significantly more resistant than 10403S since, as mentioned above, 0.4% H_2O_2 resulted in no reduction of the numbers for EGD-e (data not shown), while it caused a 5-log reduction for 10403S ($P < 0.05$; Fig. 3B).

Subsequently, we investigated the effect of growth phase on resistance against H_2O_2. When cells of WT EGD-e grown aerobically at 37°C to mid-exponential phase were challenged with 0.4% H_2O_2, CFU remained stable for both the WT and the Δ*sigB* mutant within 60 min, while when they were challenged with 3% H_2O_2, the numbers of cells were reduced to below the detection limit within 20 min (data not shown). When these cells were challenged with 1% H_2O_2, the WT EGD-e was more sensitive than the Δ*sigB* mutant (Fig. 4A). However, this sensitivity was not as pronounced as in stationary phase, with the log reduction being similar for the first 20 min of the challenge. Interestingly enough, there was no significant difference between the Δ*sigB* mutant and WT 10403S at mid-exponential phase of growth at 37°C against 0.43% H_2O_2 ($P > 0.05$; Fig. 4B). Furthermore, when 1% and 3% H_2O_2 were used, the CFU of both the WT 10403S and Δ*sigB* mutant were reduced to below the detection limit within the first 20 min.

Disk diffusion assay. Disk diffusion assays were performed with 10403S and EGD-e at both temperatures (30°C and 37°C). A statistically significant difference ($P < 0.05$) was found only with 10403S at 30°C, where the Δ*sigB* mutant showed a smaller inhibition zone than that of its isogenic WT (Table 2). No significant difference was observed with 10403S at 37°C and with EGD-e at both temperatures.

Catalase test. A catalase test was performed under all the conditions examined in this study. Under aerobic conditions and stationary phase, the Δ*sigB* mutants produced a more vigorous catalase reaction than their isogenic WT strains (Fig. 1 and 2). Under anaerobic conditions and stationary phase, the WT EGD-e showed stronger catalase activity than the Δ*sigB* mutant, which

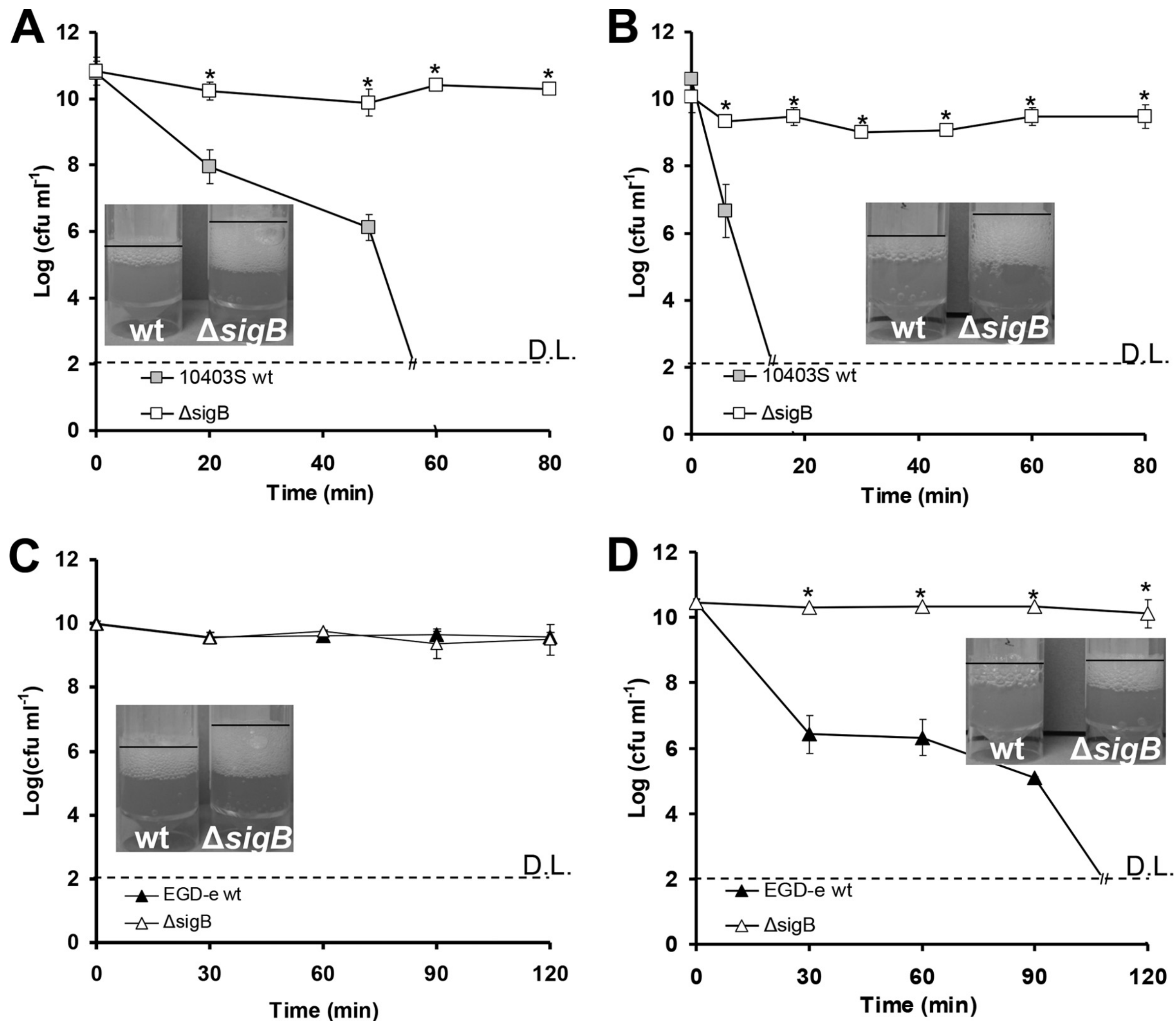

FIG 1 Cells of 10403S WT (gray boxes) and its isogenic Δ*sigB* mutant (white boxes) grown aerobically until stationary phase and challenged with 3% H_2O_2 at 30°C (A) or 37°C (B). Cells of EGD-e WT (black triangles) and its isogenic Δ*sigB* mutant (white triangles) grown until stationary phase and challenged with 3% H_2O_2 at 30°C (C) or 37°C (D). D.L. denotes the detection limit, and the asterisks denote a difference with statistical significance ($P < 0.05$). Catalase tests were performed in triplicate (one representative experiment is shown) with the addition of 100 μl of 30% H_2O_2 solution in 5-ml samples of the cultures. Cultures for catalase tests were grown under conditions similar to those used in challenge tests. The error bars represent the standard deviations.

coincides with the higher survival rate of the WT over the Δ*sigB* mutant (Fig. 3A). However, under the same conditions, the WT 10403S did not show a major difference compared to the Δ*sigB* mutant (Fig. 3B). At mid-exponential phase under aerobic conditions, the Δ*sigB* mutant had a stronger catalase test result than the WT in EGD-e (Fig. 4A), while in 10403S, the WT and Δ*sigB* mutant showed no difference in catalase activity (Fig. 4B).

Furthermore, under aerobic conditions, stationary-phase cells produced a more vigorous catalase activity test result than those grown anaerobically. In addition, under aerobic conditions, mid-exponential-phase cells showed weaker catalase activity than those at stationary phase, while EGD-e produced stronger catalase activity than 10403S under all conditions.

Catalase activity in protein extracts. These experiments were performed with stationary-phase cells of 10403S at both temperatures (30°C and 37°C) to assess intracellular catalase activity. In lysates obtained from 10403S cells grown at 37°C under aerobic conditions, the Δ*sigB* mutant was able to degrade H_2O_2 faster than its isogenic WT (Fig. 5). The results were similar with cells obtained at 30°C (data not shown). This suggests a stronger catalase reaction for the Δ*sigB* mutant at stationary phase.

DO measurements. The DO was measured in bacterial cultures grown aerobically and anaerobically (Table 3). No statistically significant differences were found between the levels of DO in the WT and Δ*sigB* mutant ($P > 0.05$) under any of the conditions studied.

Transcriptional analysis of genes contributing to oxidative stress. No statistically significant difference was observed between the Δ*sigB* mutant and WT in the transcription of any of the genes analyzed. The Δ*sigB* mutant transcript levels for *kat*, *lmo0367*, *lmo1604*, and *tpx* were 49.40%, 46.18%, 47.67%, and 45.05% of those for the WT, respectively, but in all cases, the *P* value was >0.05.

Caco-2 proliferation assays. The intracellular proliferation of the 10403S WT and Δ*sigB* mutant was assessed for 12 h (Fig. 6). Except for the first 2 h, where a significant difference was found

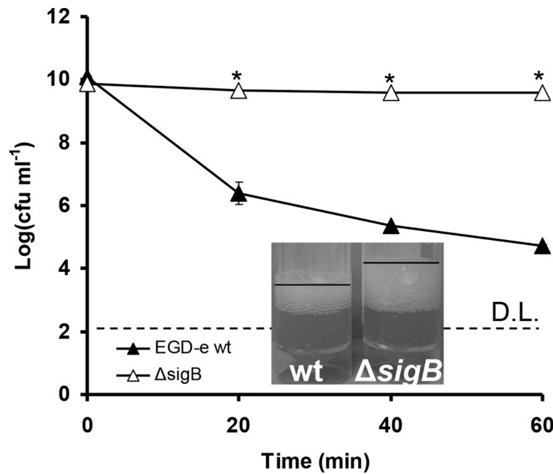

FIG 2 Cells of EGD-e WT (black triangles) and its isogenic Δ*sigB* mutant (white triangles) grown aerobically until stationary phase and challenged with 4.5% H_2O_2 at 30°C. D.L. denotes the detection limit, and the asterisks denote a difference with statistical significance ($P < 0.05$). Catalase tests were performed in triplicate (one representative experiment is shown) with the addition of 100 μl of 30% H_2O_2 solution in a 5-ml sample of the cultures. Cultures for catalase tests were grown under conditions similar to those used in challenge tests. The error bars represent the standard deviations.

($P < 0.05$), the bacterial proliferation was similar in the WT and Δ*sigB* mutant ($P > 0.05$). However, the statistically significant difference ($P < 0.05$) in the first 2 h is due to the previously well-documented lower invasion of the Δ*sigB* mutant. Introducing a 1.2-h delay in the WT curve to compensate for the lower initial numbers of the Δ*sigB* mutant results in similar proliferation curves for the two strains (Fig. 6, inset).

DISCUSSION

In the present study, we investigate the role of *L. monocytogenes* SigB in resistance against hydrogen peroxide-mediated oxidative stress. SigB is the central regulator of stress genes in Gram-positive bacteria, such as *L. monocytogenes*. It is well known that SigB plays an important role against various stresses, such as acid, heat, salt, bile acids, etc. (6–8), as well as oxidative stress (7, 9). Interestingly, despite the above-mentioned well-established role for SigB as an important factor for stress resistance, we show conclusively in two reference strains of *L. monocytogenes* (EGD-e and 10403S) that SigB confers a survival disadvantage during oxidative stress in stationary-phase cells grown aerobically (Fig. 1 and 2). The loss of SigB exerted such a dramatic effect that the Δ*sigB* mutant was more resistant to H_2O_2 by at least 7 logs compared to the WT. In general, stationary-phase cells were more resistant to H_2O_2 at 30°C than at 37°C (Fig. 1 and 2), which was also apparent with the disk diffusion results (Table 2). However, the hyperresistant phenotype of the Δ*sigB* mutant was not so pronounced in disk diffusion assays, as it was not observed in EGD-e but only slightly in 10403S at 30°C and not at 37°C. This is most probably due to the disk diffusion assays assessing inhibition of growth, rather than survival after growth, which is mainly assessed in this study.

Overall, our results show a similar behavior between *L. monocytogenes* and *B. cereus*, where the presence of *sigB* also increased sensitivity against oxidative stress (11). In contrast with the mentioned study, where only mid-exponential-phase cells were used, we assessed exponential- and stationary-phase cells. The role of SigB in *L. monocytogenes* under oxidative stress is controversial, as there are discrepancies in the results of different workers. Our results are partly in agreement with those of Moorhead and Dykes (12), where the Δ*sigB* mutant of a serotype 4c strain *L. monocytogenes* was more resistant to oxidative stress than its isogenic WT. However, no other quantitative data apart from this information were presented in this paper to allow comparisons. It was also noted that L61, a serotype 1/2a strain, did not show the above-mentioned phenotype, as the Δ*sigB* mutant and WT showed similar resistance to oxidative stress. As a result, the authors suggest that this phenotype might be serotype dependent, occurring in serotype 4c strains. However, this does not seem to be the case, since we observed this phenotype in a serotype 1/2a strain (10403S). On the contrary, Oliver et al. (18) showed that SigB plays a limited role in the resistance of *L. monocytogenes* F2365

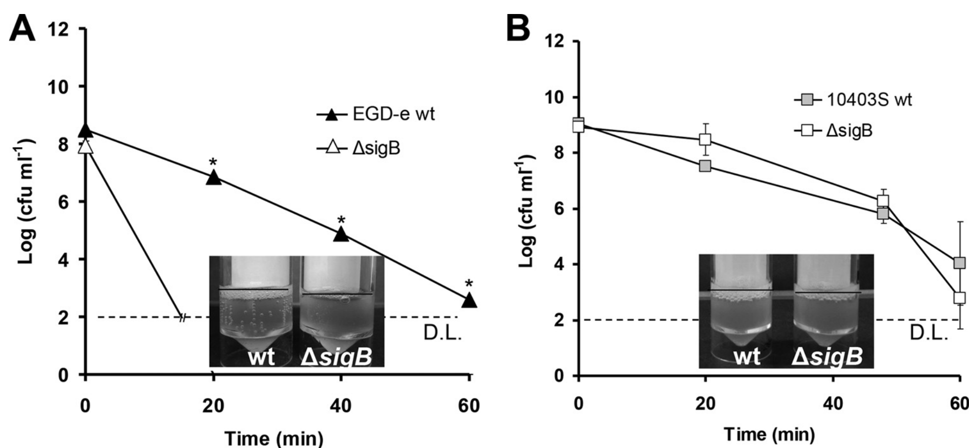

FIG 3 Cells of EGD-e (A) and 10403S (B) (WT and Δ*sigB* mutant) grown anaerobically (static in Sterilin Quickstart Universal polystyrene containers in an anaerobic chamber) at 37°C overnight and then challenged with 1% (A) and 0.4% (B) H_2O_2, respectively. D.L. denotes the detection limit, and asterisks denote a difference with statistical significance ($P < 0.05$). Catalase tests were performed in triplicate (one representative experiment is shown), with the addition of 100 μl of 30% H_2O_2 solution in a 5-ml sample of the cultures. Cultures for catalase tests were grown under conditions similar to those used in challenge tests. The error bars represent the standard deviations.

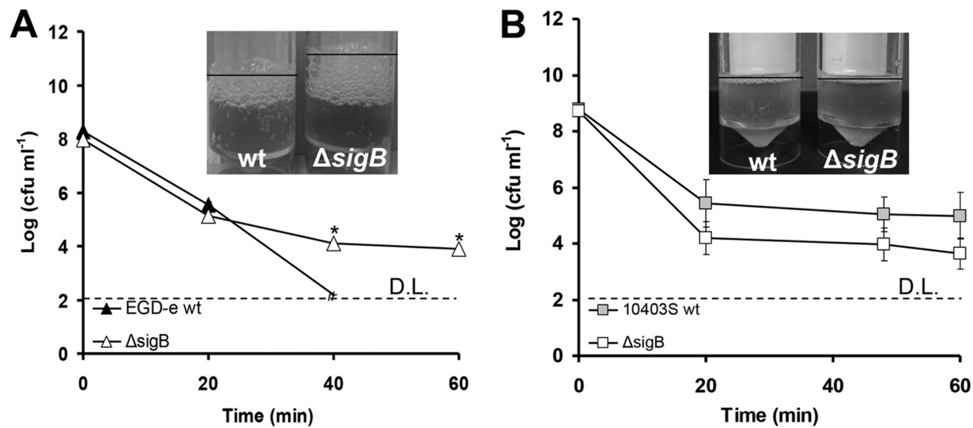

FIG 4 Cells of EGD-e (A) and 10403S (B) (WT and Δ*sigB* mutant) grown aerobically at 37°C until the mid-exponential phase of growth and then challenged with 1% (A) and 0.43% (B) H_2O_2. D.L. denotes the detection limit, and the asterisks denote a difference with statistical significance ($P < 0.05$). Catalase tests were performed in triplicate (one representative experiment is shown), with the addition of 100 μl of 30% H_2O_2 solution in 5 ml of culture. Cultures for catalase tests were grown under conditions similar to those used in challenge tests. The error bars represent the standard deviations.

against oxidative stress (18), despite the fact that this strain does not utilize an active SigB-dependent response pathway, and therefore, the influence of SigB to its stress resistance is expected to be limited anyway. Most interestingly, SigB has been shown to contribute to resistance against oxidative stress in other strains, including the one we used (10403S) in the current study (7, 9). The above-mentioned discrepancies might be due to the authors (7) using cumene hydroperoxide instead of the H_2O_2 used in our case, or due to the different length of time cultures were allowed to grow prior to experiments, as we used 18-h cultures instead of 12-h cultures. Cultures grown for a shorter length of time might have a behavior similar to that of exponential-phase cultures, where WT cells might survive better than the Δ*sigB* mutant cells. We are currently investigating the point during growth that signifies the development of the hyperresistant phenotype. However, the most possible explanation for the discrepancies might be different levels of oxygen tension in the cultures, although strain variability should not be excluded. Specifically, in most of the above-mentioned cases, small culture volumes were used prior to experiments (5 ml of BHI). The limited headspace in this setup might not have allowed sufficient levels of oxygen to dissolve in the medium.

As we demonstrate here, oxygen tension and strain variability influence greatly the H_2O_2 resistance phenotype. Under all the conditions studied (aerobic at both temperatures and anaerobic), the WT and *sigB* mutant presented similar levels of

TABLE 2 Zones of inhibition diameters for disk diffusion assays with 30% H_2O_2

	Zone of inhibition diam (mm)		
Temp (°C)	WT	Δ*sigB* mutant	*P*
10403S			
30	57	48	0.03[a]
37	49	51	0.25
EGD-e			
30	36	35	0.52
37	38	37	0.05

[a] Denotes statistical significance over 6 replicates using paired *t* test.

oxygen (Table 3), suggesting that the differences in H_2O_2 resistance between the WT and Δ*sigB* mutant could not be attributed to different oxygen levels. When grown under anaerobic conditions, and consequently with low levels of oxygen, both EGD-e and 10403S strains were considerably more sensitive than at aerobic growth. However, under anaerobic conditions, the absence of *sigB* in EGD-e resulted in an H_2O_2-sensitive phenotype (Fig. 3A). This is in contrast to what has been observed in *B. cereus*, where the H_2O_2-resistant phenotype of the Δ*sigB* mutant was retained following growth under anaerobic conditions, albeit to a lesser extent (11). However, this might be attributed to the different stage of growth affecting *sigB* expression, as experiments under anaerobic conditions in *B. cereus* were performed with mid-exponential-phase cells, while we used stationary-phase cells. As observed in *B. cereus*, the H_2O_2 resistance was lower under anaerobic than under aerobic conditions (11), as a 3-fold-lower con-

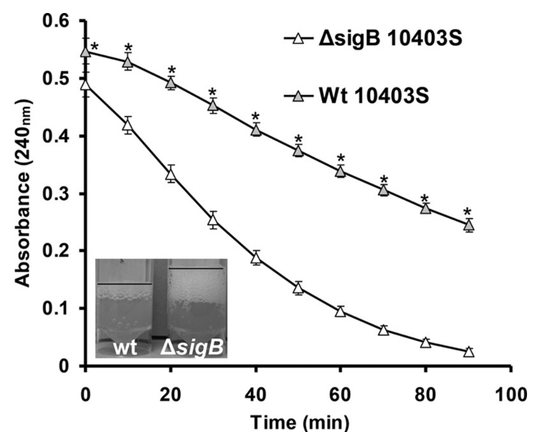

FIG 5 Absorbance at 240 nm (corresponding to concentration of H_2O_2) indicating the catalase activity of cell lysates of 10403S WT (gray triangles) and its isogenic Δ*sigB* mutant (white triangles). A more rapid reduction of the absorbance indicates more-rapid H_2O_2 degradation and therefore higher catalase activity. Cells used for lysate preparation were from the stationary phase and grown at 37°C. Each point is an average of the results from 3 biological and 3 technical replicates, while the error bars represent the standard deviations. The asterisks denote a difference with statistical significance ($P < 0.05$).

TABLE 3 Dissolved oxygen in *Listeria monocytogenes* cultures

Strain	Dissolved oxygen (mean ± SD) (mg/liter)		
	Aerobic growth		Anaerobic growth
	30°C	37°C	
EGD-e			
WT	7.25 ± 0.07	6.53 ± 0.23	0.07 ± 0.04
Δ*sigB* mutant	7.25 ± 0.08	6.57 ± 0.24	0.07 ± 0.03
10403S			
WT	7.00 ± 0.09	6.41 ± 0.12	0.06 ± 0.03
Δ*sigB* mutant	7.10 ± 0.05	6.50 ± 0.22	0.21 ± 0.19

centration of H_2O_2 achieved a higher log reduction for EGD-e anaerobically grown cells (Fig. 3A) than those grown aerobically (Fig. 1D). This is because H_2O_2 is a by-product of aerobic growth, and cellular responses against it (e.g., catalase activity) are expected to appear under aerobic conditions (11). In contrast, under anaerobic conditions, no H_2O_2 is expected to be formed, and therefore, this might result in the absence of responses to H_2O_2.

As mentioned earlier, another factor affecting *sigB* expression is the stage of growth. Cells of EGD-e WT grown aerobically to mid-exponential phase (Fig. 4A) were highly sensitive to low concentrations (1%) of H_2O_2, surpassing in sensitivity even those grown anaerobically (Fig. 3A). Furthermore, as expected, the role of SigB seemed to be limited at mid-exponential phase (10), as there was only a minor difference between the Δ*sigB* mutant and WT EGD-e over the first 20 min (Fig. 4A). Following 40 min of H_2O_2 challenge, the Δ*sigB* mutant appeared slightly more resistant than the WT, which is consistent with the catalase activity tests, confirming that SigB confers a survival disadvantage for the cells grown aerobically, even if its influence is minimal. However, no statistically significant results ($P > 0.05$) were obtained with 10403S cells grown aerobically until the exponential phase (Fig. 4B), while the WT appeared slightly more resistant than the Δ*sigB* mutant to 0.43% H_2O_2.

In general, we found that the survival results could be explained solely by the catalase tests, with cells producing more vigorous bubbling being more resistant to oxidative stress. At stationary phase under aerobic conditions, in all cases, the Δ*sigB* mutant showed stronger catalase activity upon addition of 30% H_2O_2 than the WT (Fig. 1 and 2). To assess the intracellular catalase activity (H_2O_2 degradation in protein extracts; Fig. 5) we found that it corresponded well with the overall catalase activity of live cells. This confirmed beyond a doubt that mutations in *sigB* result in enhanced catalase activity and oxidative stress resistance under aerobic conditions, which coincides with what has been described in *B. cereus* (11).

To identify possible molecular mechanisms behind the higher catalase activity and the hyperresistance of the Δ*sigB* mutant to oxidative stress, we looked at the transcription of specific genes that protect against oxidative stress and H_2O_2. Interestingly enough, despite the difference between the catalase activities of the WT and Δ*sigB* mutant, we found no difference in the transcription of the *kat* (*lmo2785*) gene encoding catalase between the Δ*sigB* mutant and the WT. Since, to our knowledge, *L. monocytogenes* possesses only one catalase gene, these data suggest that differential expression of the catalase gene might occur posttranscriptionally; alternatively, differential transcription might occur earlier during growth. In addition, we investigated the transcription of thiol peroxidase (*tpx* or *lmo1583*), 2-cys peroxiredoxin (*lmo1604*), and a hypothetical iron peroxidase (*lmo0367*) that could potentially confer protection against oxidative stress. Interestingly, Lmo1604 has been found to be negatively regulated by σ^B but in the presence of salt (16). However, similarly to *kat*, all these genes showed similar transcription in the WT and Δ*sigB* mutant.

Since oxidative stress is one of the most important stresses occurring within the phagolysosome, it could be expected that this hyperresistance of the Δ*sigB* mutant to oxidative stress might translate into higher intracellular survival. However, this is not the case, as we show here (Fig. 6), and as was demonstrated previously in other studies (5, 19, 20). We saw a lower level of invasion for the Δ*sigB* mutant, which has been documented by other workers (5) and occurs due to lower expression of internalins *inlA* and *inlB* (21), but not major differences during the proliferation stage. However, the explanation for this lies in the fact that following invasion, *L. monocytogenes* does not utilize the SigB intracellularly, as was shown previously (22), and as a result, Δ*sigB* mutants do not show any difference compared to the WT in proliferation assays (20, 22) or in systemic spread in the guinea pig model (19). Therefore, SigB expression occurs in the gut at the extracellular stage of infection, as it is important for internalin expression, but it is subsequently abolished during the intracellular stage. It could be said that *L. monocytogenes* tries to mimic the behavior of the Δ*sigB* mutant intracellularly. A logical explanation for this behavior is that the abolishment of SigB expression intracellularly could lead to higher resistance toward oxidative stress, which occurs in the phagolysosome. However, there is no satisfactory explanation on how *L. monocytogenes* is able to prevent the upregulation of SigB intracellularly, since the phagolysosome is a particularly stressful environment. We currently investigate this further as well as the environmental factors affecting this behavior.

This study illustrates that although *sigB* is important for general stress resistance, an important deviation to this pattern occurs in the case of oxidative stress under aerobic conditions at stationary phase. In addition, we demonstrate that oxygen tension and growth phase can affect significantly this phenotype in *L. monocy-*

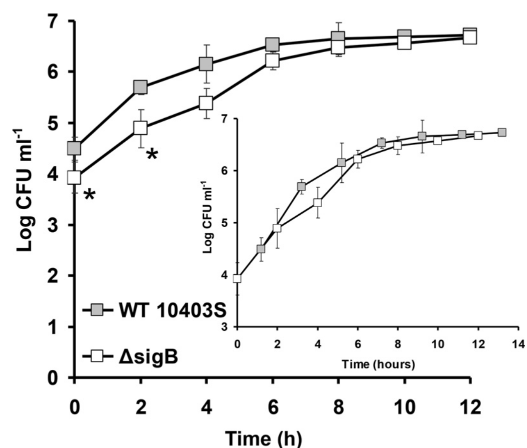

FIG 6 Proliferation assays of cells of *L. monocytogenes* 10403S WT and its isogenic Δ*sigB* mutant in Caco-2 epithelial cells. The asterisks denote a difference with statistical significance ($P < 0.05$). The inset shows both proliferation curves of 10403S WT and the Δ*sigB* mutant overlaid with WT curve delayed by 1.2 h. The error bars represent the standard deviations.

togenes. An attempt to identify the molecular mechanism behind this phenotype was unsuccessful, and future work will focus in this direction.

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