

Listeria monocytogenes PerR Mutants Display a Small-Colony Phenotype, Increased Sensitivity to Hydrogen Peroxide, and Significantly Reduced Murine Virulence

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Deletion of *perR* in *Listeria monocytogenes* results in a small-colony phenotype ($\Delta perR_{sm}$) that is slow growing and exhibits increased sensitivity to H₂O₂. At a relatively high frequency, large-colony variants ($\Delta perR_{lg}$) arise, which are more resistant to H₂O₂ than the wild-type and ultimately dominate the culture. Transcriptional analysis revealed that the *kat* gene (catalase) is up-regulated in both types of mutants and that the highest level is apparent in $\Delta perR_{sm}$ mutants, demonstrating PerR regulation of this gene. Overexpression of the catalase gene in the wild-type background resulted in a slower-growing strain with a smaller colony size similar to that of $\Delta perR_{sm}$. By combining a bioinformatic approach with experimental evidence, other PerR-regulated genes were identified, including *fur*, *lmo0641*, *fri*, *lmo1604*, *hemA*, and *trxB*. The transcriptional profile of these genes in both mutant backgrounds was similar to that of catalase in that a higher level of expression was observed in $\Delta perR_{sm}$ than in the wild type or $\Delta perR_{lg}$. Murine studies revealed that the virulence potential of the $\Delta perR_{sm}$ mutant is substantially reduced compared to that of the wild-type and $\Delta perR_{lg}$ strains. Collectively, the data demonstrate that the $\Delta perR_{sm}$ mutant represents the true phenotype associated with the absence of PerR, which is linked to overexpression of regulated genes that negatively affect bacterial homeostasis both in vitro and in vivo. A subsequent secondary mutation occurred at a high frequency, which resulted in phenotypic reversion to a large-colony phenotype with increased fitness that may have obstructed the analysis of the role of PerR in the physiology of the bacterial cell.

Reactive oxygen species (ROS), such as hydroxyl radicals, superoxide anions (O₂⁻), and hydrogen peroxide (H₂O₂), are an inevitable consequence of aerobic growth (35). If excessive amounts are present, these highly reactive compounds can damage DNA, proteins, and lipid membranes (26). Aerobic and facultative bacteria have evolved highly sophisticated inducible responses in order to survive these deleterious effects. The important ROS-inducible systems include superoxide dismutase, catalase (Kat), alkyl hydroperoxide reductase (AhpCF), and proteins required for protection and repair (22).

In aerobically growing cells, specific transcriptional regulators are required to activate defenses against the significant amounts of O₂⁻ and H₂O₂ which are generated (34). In *Escherichia coli* one system is mediated by OxyR, a peroxide-sensing regulator with a regulon which includes genes involved in peroxide metabolism and protection (including *ahpCF*, *katG*, and *dps*) (34). This regulator is widely distributed in most gram-negative and some gram-positive bacteria. However, in *Bacillus subtilis* and *Staphylococcus aureus* protection against ROS is mediated principally by PerR (17, 22, 24). The PerR regulon of *B. subtilis* has been well characterized and includes Kat, AhpCF, MrgA (DNA protection), the HemA operon (heme biosynthesis), and PerR itself (7, 8, 17, 35). These systems mount a response that protects cells when they are challenged with peroxide stress. Unlike OxyR, PerR is a metalloregulatory protein consisting of two metal-binding domains.

The zinc ion has a structural role, with either Fe or Mn complexed in a regulatory role (23).

Interestingly, Fur (iron uptake and homeostasis) (2) and ZosA (zinc uptake during peroxide stress) (20) also form part of the PerR regulon in *B. subtilis*. Metal ion homeostasis can also have a significant impact on the effect of ROS. For example, unregulated uptake of iron, like that in a Fur mutant, can lead to the formation of toxic hydroxyl radicals (1, 40). As a result, metal ion uptake must be tightly regulated and is controlled by four main metalloregulatory proteins in *B. subtilis*, namely, Fur, PerR, Zur (zinc uptake and homeostasis), and MntR (manganese uptake and homeostasis) (25, 39).

Listeria monocytogenes is a gram-positive pathogen which can cause serious infections in susceptible individuals (47). The ability to respond to stress plays an important role in the virulence of this pathogen (5, 11, 33, 46). *L. monocytogenes* is extremely aerotolerant and hence must be able to resist the action of ROS. A homologue of each of the four main metalloregulators in *B. subtilis* has been identified in *Listeria*. Establishment of an infection requires tightly controlled gene regulation, and any disruption of the series of events may decrease the fitness and result in a less virulent strain (21, 42, 43). In a previous study we found that eliminating PerR resulted in a mutant which was more resistant to the action of H₂O₂ but was marginally less virulent than the wild type (42). We also observed that creation of the PerR mutant resulted in two phenotypically distinct colony types, one much smaller than the wild type and one whose size was equivalent to that of the wild type. In that study, we did not discriminate between colony types in further analyses, and, given the higher growth rates of

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TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Characteristics	Source or reference
<i>Listeria monocytogenes</i> strains		
EGDe	Serotype 1/2a	W. Goebel
EGDe $\Delta perR_{sm}$	Wild type with a deletion in <i>perR</i> , small-colony phenotype	42
EGDe $\Delta perR_{lg}$	Wild type with a deletion in <i>perR</i> , phenotypically reverted to large-colony phenotype	42
EGDe-NICE	EGDe derivative with the <i>nisRK</i> genes integrated on the chromosome; expression host for nisin-induced expression system	Sheila Ryan
EGDe-NICE(pNZ8048)	EGDe-NICE containing the cloning vector pNZ8048	This study
EGDe-NICE(pNZ8048- <i>kat</i>)	EGDe-NICE containing pNZ8048 expressing <i>kat</i> under the control of the nisin-induced promoter	This study
Plasmids ^a		
pNZ8048	Cm ^r ; nisin-inducible expression vector	14
pNZ8048- <i>kat</i>	pNZ8048 with <i>kat</i> under the control of the <i>nisA</i> promoter	This study
pPTPL	Tet ^r ; promoter probe vector	36
pPTPL- <i>kat</i>	pPTPL with the <i>kat</i> promoter driving the expression of β -galactosidase	This study
pPL2	Cm ^r ; integrates at PSA phage attachment site within a tRNA ^{Arg} gene on the chromosome	29
<i>perR</i> ⁺ _{lg}	$\Delta perR_{lg}$ with a full copy of the <i>perR</i> gene integrated at tRNA ^{Arg} - <i>attB</i>	This study
<i>perR</i> ⁺ _{sm}	$\Delta perR_{sm}$ with a full copy of the <i>perR</i> gene integrated at tRNA ^{Arg} - <i>attB</i>	This study
Primers ^b		
KatF	TTACCGCGAATTCGGGGCT	
KatR	ACAAACGGTCTAGAGAAGGA	
pKatF	CTAAGAGATCTCTTTTATAAGC	
pKatR	CGTTAAATTTTCTAGATGT	
NZKatF	ATACCCATGGCAGATAGAAA	
NZKatR	ACTCCAACCTTCTAGACCAA	
CompF	CATTCGGATCCATATTTAGAGC	
CompR	CATATCACTGCAGCAACTATCT	

^a Tet^r, tetracycline resistance; Cm^r, chloramphenicol resistance.

^b Restriction sites incorporated into primer sequences are underlined.

the large-colony phenotype, it is likely that in this initial work we analyzed the phenotype of the large-colony mutant.

In the current study, we established that the small-colony phenotype represents the “true” phenotype associated with loss of PerR and that the large-colony revertant represents a subsequent, unidentified mutation. We also examined the consequences of PerR deletion for bacterial fitness and identified a number of PerR-regulated genes in *Listeria*. We analyzed the expression of these genes in the two mutant (small- and large-colony) backgrounds. The H₂O₂ sensitivity of both mutants was also examined because of the well-defined role of PerR in defense against peroxide stress. The ability of these mutants to establish an infection compared to the ability of the wild type was analyzed using a mouse model of infection. Overall, this work demonstrated that in contrast to the findings of previous reports, *L. monocytogenes* PerR mutants are actually less resistant to peroxide stress and that the ability to cause infection in the mouse model is dramatically affected.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *E. coli* strains were grown in Luria-Bertani medium, and *L. monocytogenes* strains were grown in brain heart infusion broth (BHI) (Oxoid) at 37°C. When appropriate, antibiotics (Sigma Chemical Company, St. Louis, Mo.) were used in *L. monocytogenes* cultures at the following concentrations: tetracycline, 10 μ g/ml; and chloramphenicol, 10 μ g/ml, unless otherwise stated. For solid media agar was added to a concentration of 1.5%. Nisin (Sigma Chemical Company, St. Louis, Mo.) was solubilized in dimethyl sulfoxide and acetic acid and was added to sterilized media.

DNA manipulation. Gel extraction was performed using a QIAGEN gel extraction kit (QIAGEN). Plasmid DNA was isolated using a QIAGEN QIAprep

Spin Miniprep kit (QIAGEN). T4 DNA ligase, PCR reagents, and restriction enzymes were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and were used according to the manufacturer's instructions. PCRs were performed using a Hybaid (Middlesex, United Kingdom) PCR express system, and products were cloned into pPTPL and pNZ8048 and sequenced using plasmid-specific primers (Lark Technologies Inc., Essex, United Kingdom). Oligonucleotide primers (Table 1) for PCR were synthesized by Sigma-Genosys Biotechnologies.

Growth curves. Overnight cultures were centrifuged (12,000 rpm for 5 min), washed, and resuspended in an equal volume of one-quarter-strength Ringer's solution (Merck). A 2% inoculum was added to 10 ml BHI. Two hundred microliters was added to a 96-well plate, and growth was determined automatically at 600 nm using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, Calif.) for 24 h at 37°C. Viability was assessed by plate counting. Overnight cultures prepared as described above were inoculated (2%) into 10 ml BHI. Bacterial counts were determined at this stage (time zero) and then every 3 h for 12 h. Briefly, 100 μ l was removed, serially diluted, and plated onto BHI agar plates, which were incubated at 37°C overnight. The wild-type, $\Delta perR_{sm}$, and $\Delta perR_{lg}$ strains were passaged for nine consecutive days. Initially, overnight cultures (2%) were inoculated into BHI. These cultures were passaged into fresh BHI (passage 1) on the following day and for the next 8 days. A sample was removed from each passage, serially diluted, and plated onto BHI agar plates for enumeration. Overnight cultures (2%) were also inoculated into BHI with nisin (45 μ g nisin powder/ml), and growth was monitored automatically as described above.

Complementation of deletion mutants. pPL2 was used for complementation of $\Delta perR_{lg}$ and $\Delta perR_{sm}$. This plasmid is a site-specific phage integration vector which integrates within a tRNA^{Arg} gene on the chromosome. The entire *perR* gene and flanking regions were amplified from the *L. monocytogenes* EGDe genome using the proofreading enzyme *Pwo* DNA polymerase (Roche). Primers CompF and CompR were designed to incorporate BamHI and PstI restriction sites, respectively. The resulting PCR product was gel extracted, digested with the enzymes described above, and cloned into similarly digested vector pPL2. This construct was electroporated into DH5 α , and transformants were selected using Luria-Bertani plates with 15 μ g of chloramphenicol/ml. Plasmids were

extracted using a QIAGEN QIAprep Spin Miniprep kit. The presence of the correct insert was confirmed by sequencing (Lark) using the T3 and T7 primers. Plasmid preparations were ethanol precipitated and resuspended in 5 μ l (final volume) of elution buffer (10 mM Tris-Cl, pH 8.5). This preparation was electroporated into competent Δ *perR*_{lg} and Δ *perR*_{sm} cells, and transformants were selected on BHI agar plates containing 7.5 μ g of chloramphenicol/ml. pPL2 with no insert was transformed into mutant cells as a control. Integration at the correct location on the chromosome was confirmed by PCR using a primer that annealed 5' of the integration site and a primer that annealed to the cloned insert.

Hydrogen peroxide sensitivity assays. Overnight cultures were inoculated into fresh BHI and grown to the early log phase (optical density at 600 nm [OD₆₀₀], ~0.20). Cells were harvested and washed with one-quarter-strength Ringer's solution. Hydrogen peroxide was added to BHI to a final concentration of 50 mM (bactericidal), and 1 ml was used to resuspend the pellets. Samples were taken every 30 min for 2 h, and survivors were determined by dilution in Ringer's solution and plating onto BHI agar. Overnight cultures (2%) were also inoculated into BHI with 22 mM H₂O₂ (bacteriostatic), and growth was monitored automatically as described above. For the disk assay overnight cultures were inoculated into fresh BHI and grown to the early log phase (OD₆₀₀, ~0.2). Four hundred microliters was added to 4 ml of sloppy agar (0.75%), poured onto 20 ml of BHI agar (1.5%), and left to solidify. A sterile blank disk was placed in the center of the agar, to which 20 μ l of 33% hydrogen peroxide was added. The plates were incubated at 37°C overnight.

Catalase activity assay. Strains grown statically overnight at 37°C were resuspended in 1/20 the original culture volume and lysed using a mini bead beater. Debris was pelleted by centrifugation at 12,000 \times g at 4°C for 10 min. The total protein concentration was determined by the method of Lowry et al. (32) following protein precipitation with 10% trichloroacetic acid. Catalase activity was determined using the method described by Beers and Sizer (4). Briefly, 0.5 ml of H₂O₂ (59 mM H₂O₂ freshly diluted in 50 mM potassium phosphate buffer, pH 7.0) was added to 1 ml of the crude extract, and the absorbance at 240 nm was measured every 15 s for 1 min. The specific activity of catalase (expressed in micromoles of H₂O₂ decomposed per minute per milligram of total protein) was calculated as follows:

$$\frac{1,000 \times \Delta_{240}/\text{min (average)}}{43.6 \times \text{mg of protein/ml of reaction mixture}}$$

RNA extraction. RNA was extracted from log- and stationary-phase cells using the Macaloid method (41). Briefly, cells were spun and lysed using a combination of glass beads, 10% sodium dodecyl sulfate, and a mini bead beater. RNA was isolated from the cellular debris using Macaloid clay and phenol-chloroform (Sigma Chemical Company, St. Louis, Mo.). The RNA was purified further by extraction with phenol-chloroform and chloroform-isoamyl alcohol (Sigma Chemical Company, St. Louis, Mo.). When necessary, the RNA was treated with DNase using a DNA free kit (Ambion). The absence of contaminating DNA was confirmed using control primers. For overexpression studies RNA was isolated from EGDe-NICE(pNZ8048-*kat*) and EGDe-NICE(pNZ8048) (control) following nisin induction. Cultures were grown to an OD₆₀₀ of approximately 0.2 and preinduced with 4.5 μ g of nisin powder/ml for 1 h. This was followed by induction with 45 μ g of nisin powder/ml for 30 min. RNA was isolated at this stage.

Transcriptional analysis. The RNA was converted to cDNA using Expand reverse transcriptase and random primer p(dN)₆ (Roche). *Kat*-specific primers were used to analyze the cDNA, and samples were taken at regular intervals and electrophoresed on agarose gels. Primers for the *mmA* gene (15) were used as controls to ensure that equal-intensity cDNA was added to all PCR mixtures. The promoter of the *Kat* gene was amplified using pKatF (incorporating a BglIII site) and pKatR (incorporating an XbaI site). The resulting PCR product was digested with the BglIII and XbaI restriction enzymes and cloned into similarly digested pPTPL. Electroporation of *L. monocytogenes* with wild-type, Δ *perR*_{sm}, and Δ *perR*_{lg} backgrounds with this construct was performed by using protocols outlined by Park and Stewart (38). β -Galactosidase assays were performed as described by Miller, with modifications described by Israelsen et al. (27). Briefly, cells were spun and resuspended in LacZ buffer. Cells were lysed by adding chloroform and 0.1% sodium dodecyl sulfate and were incubated at 37°C. The reaction was started by addition of *o*-nitrophenyl- β -D-galactopyranoside (Sigma Chemical Company, St. Louis, Mo.). Upon development of a yellow color the reaction was stopped using NaCO₃. β -Galactosidase activity was calculated using spectrophotometric data.

Overexpression of catalase. The catalase gene was amplified from *L. monocytogenes* EGDe using NZKatF (incorporating the *Kat* start codon and an NcoI site) and NZKatR (incorporating the *Kat* stop codon and an XbaI site). The

resultant PCR product was digested with the NcoI and XbaI restriction enzymes and cloned into similarly digested pNZ8048. This resulted in generation of a fusion between the nisin-inducible *nisA* promoter on pNZ8048 and the *kat* gene. This event was confirmed by sequence analysis. The construct was electroporated into a wild-type strain carrying a copy of the *nisRK* genes (EGDe-NICE) on the chromosome, which generated EGDe-NICE(pNZ8048-*kat*). As a control, pNZ8048 was also introduced into this wild-type background to generate EGDe-NICE(pNZ8048).

Bioinformatics. A number of potential PerR-regulated genes were identified using the search pattern function on the listlist homepage (<http://genolist.pasteur.fr/listlist/>). The PerR box (target DNA sequence to which PerR has been shown to bind in vitro) from *B. subtilis* (16) was used to search the *Listeria* genome. Searches were restricted to within 350 bp upstream of the start codon. Members of the PerR regulon in *B. subtilis* are likely candidates and were considered regardless of the number of mismatches with the consensus sequence. Subsequent reverse transcription (RT)-PCR analysis could confirm if identified genes were PerR regulated.

Virulence assay. Overnight cultures were centrifuged, washed once with phosphate-buffered saline (PBS), resuspended, and subsequently diluted in PBS. In vivo survival was determined by inoculating 8- to 12-week old BALB/c mice intraperitoneally with 3 \times 10⁵ CFU in 200 μ l PBS. The mice were euthanized on the appropriate day postinfection, and bacteria were harvested from the spleen or liver by homogenization of organs in PBS, serial dilution of the organ homogenates on BHI agar, and incubation overnight at 37°C.

RESULTS

Physiological analysis of mutants. In a previous study we observed that disruption (using pORI19 plasmid integration) or deletion (splicing by overlap extension procedure [SOEing]) of *perR* resulted in a strain that produced much smaller colonies on BHI agar than the wild type (42). However, interspersed among the small colonies were larger colonies whose sizes were similar to those of the wild type (Fig. 1A). Subsequent PCR analysis confirmed that there was a *perR* deletion in both colony types, suggesting that the larger colonies did not represent genotypic revertants or contamination with wild-type cells (Fig. 1B).

The growth of the Δ *perR*_{sm} and Δ *perR*_{lg} mutants in BHI (pH 7.0) at 37°C was compared to that of the wild type (Fig. 1C). Under these conditions no difference in either the exponential or stationary phase was detected for the Δ *perR*_{lg} mutant. However, a significant growth defect was observed for the Δ *perR*_{sm} mutant; this mutant had a lower growth rate, and the final numbers of cells were lower. In order to determine which mutant represents the "primary" PerR mutant, we selected a single colony of each type and grew it in BHI for 9 days (Fig. 2). The colony sizes of the wild type and the Δ *perR*_{lg} mutant remained constant throughout the experiment. However, the OD values and plate counts for the Δ *perR*_{sm} mutant were lower than those for either the wild type or the Δ *perR*_{lg} mutant after 24 h, and only small colonies were recovered. However, on subsequent days the appearance of wild-type-sized colonies was observed with increasing frequency. By day 6 more than 80% of the colonies were large (Δ *perR*_{lg}), and the large-colony variants remained the dominant population in subsequent transfers. The original PerR deletion event was confirmed in these colonies, suggesting that the Δ *perR*_{lg} phenotype is a result of a secondary mutation which alleviates the growth defect associated with the initial PerR mutation event.

Hydrogen peroxide sensitivity assay. Increased resistance to peroxide stress has been reported to be associated with the loss of PerR in a number of bacteria (6, 8, 24, 28, 43). Indeed, in a

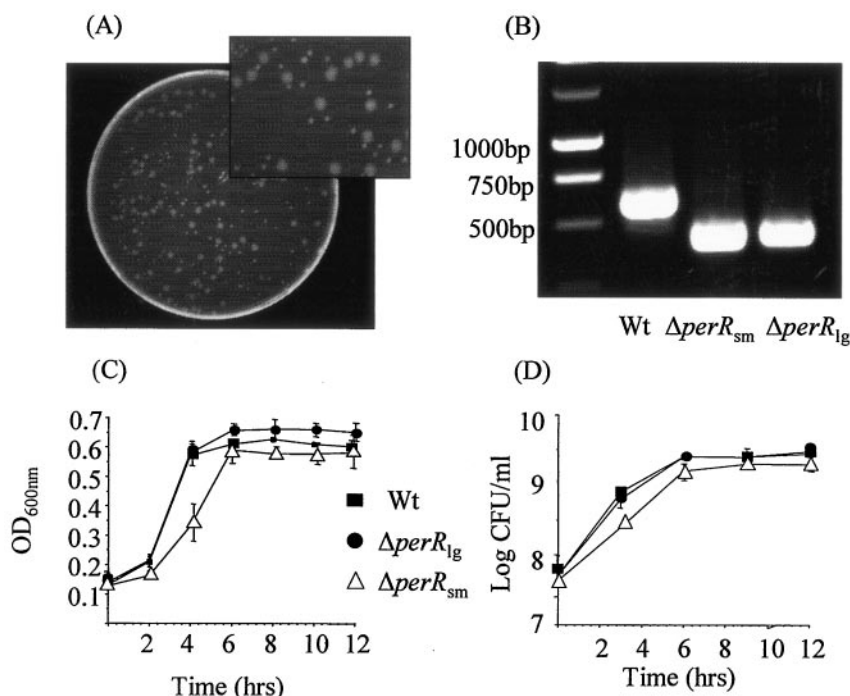


FIG. 1. (A) Deletion of the *perR* gene by the SOEing procedure in *Listeria* resulted in two colony sizes. (B) Confirmation of the deletion event in both colony types by PCR using primers located outside the deleted region. A wild-type (wt) colony was used as a control. (C) Growth of the wild type, the $\Delta perR_{sm}$ mutant, and the $\Delta perR_{lg}$ mutant in BHI at 37°C as determined by measuring the OD₆₀₀. (D) Growth of the wild type, the $\Delta perR_{sm}$ mutant, and the $\Delta perR_{lg}$ mutant in BHI at 37°C as determined by plate counting. The error bars indicate the standard deviations from the means of triplicate experiments.

previous study we found that plasmid disruption of *perR* in *L. monocytogenes* resulted in a strain that was able to respond to peroxide stress better than the wild type. This was later confirmed with a deletion mutant (42). However, in hindsight, we did not discriminate between the original small-colony mutant and the large-colony revertant in that study. To resolve this problem, we compared the abilities of the wild type, a $\Delta perR_{lg}$ mutant, and a $\Delta perR_{sm}$ mutant to respond to hydrogen peroxide. At 50 mM H₂O₂, the levels of both the wild type and the $\Delta perR_{sm}$ mutant declined significantly over a 90-min period (Fig. 3A). The decline associated with the $\Delta perR_{sm}$ mutant was much more rapid than that associated with the wild type, indicating that there was increased sensitivity to H₂O₂. However, not only was the $\Delta perR_{lg}$

mutant able to tolerate this level of H₂O₂, but some growth was observed (Fig. 3A). Furthermore, at 22 mM H₂O₂, which is inhibitory for the wild type, the $\Delta perR_{lg}$ mutant was capable of growth, albeit with an extended lag phase (Fig. 3B). The $\Delta perR_{sm}$ mutant was not capable of growth under these conditions, confirming that it is more sensitive to peroxide stress than the $\Delta perR_{lg}$ mutant. The H₂O₂ responses of the two mutants compared to the wild type were also assessed using a disk assay. The zones of inhibition observed for the $\Delta perR_{sm}$ mutant were larger than those observed for both the wild type and the $\Delta perR_{lg}$ mutant, indicating increased sensitivity to this stress. For the $\Delta perR_{lg}$ mutant the zones of inhibition were smaller than the zones of inhibition for the wild type, indicating that there was increased H₂O₂ resistance. Interestingly, reintroduction of a complete copy of the *perR* gene using the pPL2 plasmid restored the H₂O₂ response of both mutants to wild-type levels, which confirmed the phenotypes of these mutants (Fig. 3C). Thus, the increased H₂O₂ tolerance reported previously to be representative of a $\Delta perR$ mutant (42) did not reflect the true phenotype; rather, it reflected the phenotype of the $\Delta perR_{lg}$ revertant, which was associated with a secondary mutation. We concluded that the true *perR* phenotype is actually that of the $\Delta perR_{sm}$ mutant (i.e., increased H₂O₂ sensitivity).

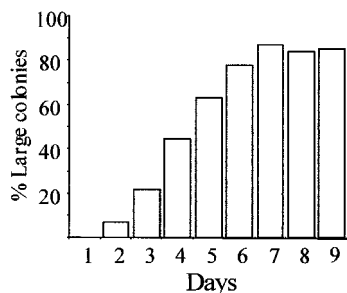


FIG. 2. Wild type, $\Delta perR_{sm}$ mutant, and $\Delta perR_{lg}$ mutant were passaged for nine consecutive days in BHI at 37°C. Data for the wild type and the $\Delta perR_{lg}$ mutant are not shown for purposes of clarity. While the graph represents one experiment, the experiment was repeated two more times, and similar results were obtained.

Transcriptional analysis. One of the principal differences between the $\Delta perR_{sm}$ and $\Delta perR_{lg}$ mutants is in their abilities to respond to H₂O₂ stress. Since catalase is the principal detoxifier of H₂O₂, we examined the expression of the *kat* gene in both mutants using RT-PCR and promoter analysis. RNA was extracted from aerobically grown wild-type, $\Delta perR_{sm}$, and $\Delta perR_{lg}$ cells in both the log and stationary phases. Interestingly,

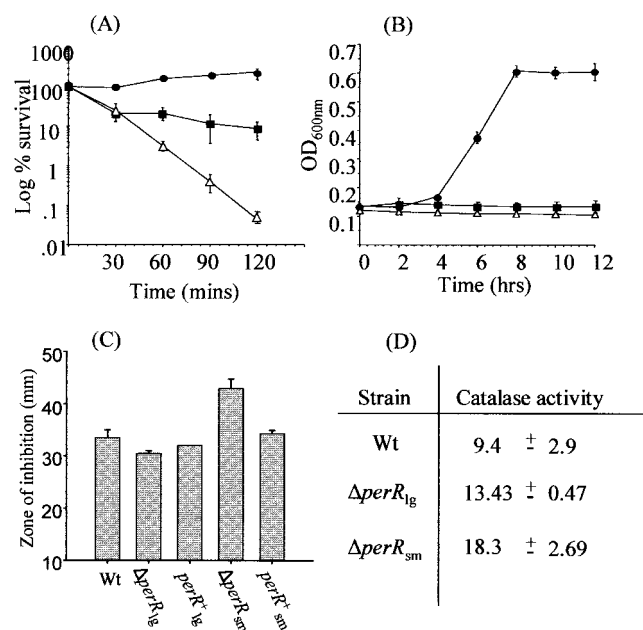


FIG. 3. (A) Effect of 50 mM hydrogen peroxide on EGDe (■) and the $\Delta perR_{lg}$ (●) and $\Delta perR_{sm}$ (△) mutants. The cells were in the exponential growth phase. The error bars indicate the standard deviations from the means of triplicate experiments. (B) Growth of *L. monocytogenes* EGDe (wild type) (■) and the $\Delta perR_{lg}$ (●) and $\Delta perR_{sm}$ (△) mutants in BHI containing 22 mM hydrogen peroxide. (C) Resistance of the wild type (Wt), the $\Delta perR_{lg}$ mutant, the $\Delta perR_{sm}$ mutant, and complemented strains to H_2O_2 , as assessed using a disk assay. (D) Levels of catalase activity in the wild-type, $\Delta perR_{lg}$, and $\Delta perR_{sm}$ strains. The catalase specific activities are expressed in micromoles of H_2O_2 decomposed per minute per milligram of total protein. Standard deviations were calculated from the means ($n = 2$). Similar results were obtained in two independent experiments.

expression of the gene was significantly up-regulated in both mutants compared to the wild type, and the highest level was apparent in the $\Delta perR_{sm}$ mutant. The results were confirmed using real-time PCR analysis (data not shown). This finding was obtained with both log-phase cells (Fig. 4A) and stationary-phase cells (Fig. 4B).

These results were subsequently confirmed using promoter studies. The *kat* promoter regions from the wild type and both mutants were cloned into the promoter probe vector pPTPL and reintroduced into the appropriate background for further analysis. β -Galactosidase activity was measured for all three strains under the conditions used for RNA extraction. The results confirmed that *kat* expression was elevated in both mutant backgrounds in log-phase cells (Fig. 4A) and stationary-phase cells (Fig. 4B). As with the RT-PCR results, a greater degree of up-regulation was observed in the $\Delta perR_{sm}$ strain. The *kat* promoter region was sequenced in order to identify any changes which might explain this altered expression, but no differences were identified. Subsequent sequencing of the entire *kat* gene in all three strains did not reveal any alteration, suggesting that the cause of this change in expression lies at a different locus.

Overexpression of catalase. To determine whether the increased expression of *kat* observed in the $\Delta perR_{sm}$ mutant could be responsible for the small-colony phenotype and the

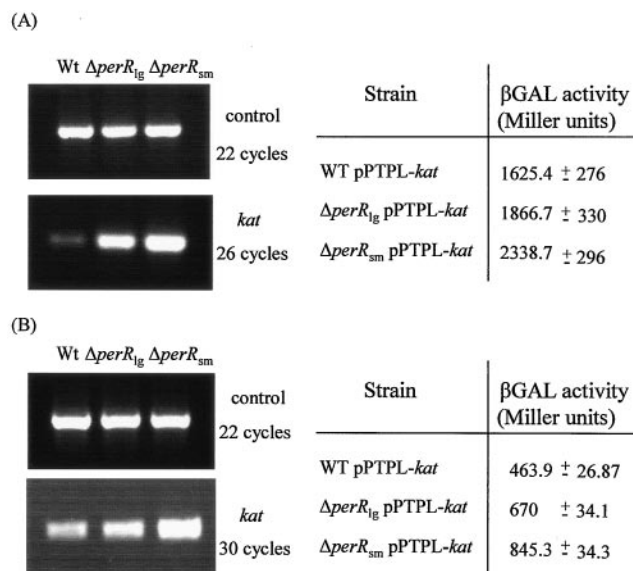


FIG. 4. RT-PCR analysis of gene expression. Control primers were used to ensure that the concentrations of template cDNAs were equal. Samples were removed at various PCR cycles and visualized on agarose gels. Transcription data were confirmed by promoter studies. Levels of β -galactosidase (β GAL) activity were calculated under the conditions used for RNA extraction. (A) Log-phase cells. (B) Stationary-phase cells. Wt, wild type.

increased peroxide sensitivity, *kat* was cloned into the expression plasmid pNZ8048, which placed the gene under control of the nisin-inducible P_{nis} promoter using the NICE (nisin-controlled expression) system (12, 14). RT-PCR analysis was used to confirm that in the presence of the nisin inducer EGDe-NICE containing pNZ8048-*kat* had substantially higher levels of catalase than the control EGDe-NICE strain containing pNZ8048 with no insert (Fig. 5A). It was observed that EGDe-NICE(pNZ8048-*kat*) had a slightly slower growth rate than the control in the presence of nisin (Fig. 5B). This growth defect was similar to that seen with the $\Delta perR_{sm}$ mutant. Induction with nisin also led to smaller colonies on BHI agar plates for EGDe-NICE(pNZ8048-*kat*) after 24 h (Fig. 5C). However, unlike those of the $\Delta perR_{sm}$ mutant, these colonies eventually reached the wild-type size. The control strain always produced wild-type-sized colonies. We also found that overexpression of catalase led to slightly decreased resistance to H_2O_2 compared to the control, as determined by a disk diffusion assay (data not shown). Therefore, many, although not all, of the phenotypes associated with the $\Delta perR_{sm}$ mutation can be attributed to the increased expression of catalase.

Bioinformatic analysis of the PerR regulon. We were interested in determining whether the up-regulation of *kat* was unique to this gene or whether there was also up-regulation of other members of the PerR regulon. We used the consensus PerR box (TTATAATnATTATAA) defined in *B. subtilis* (16) to search the *L. monocytogenes* EGDe genome for potential members of the PerR regulon. This led to identification of a large number of candidates. Only one gene, *fur*, was identified as having a PerR box identical to that in the *B. subtilis* consensus sequence. Fourteen sequences with two mismatches were identified, and many genes were likely candidates for

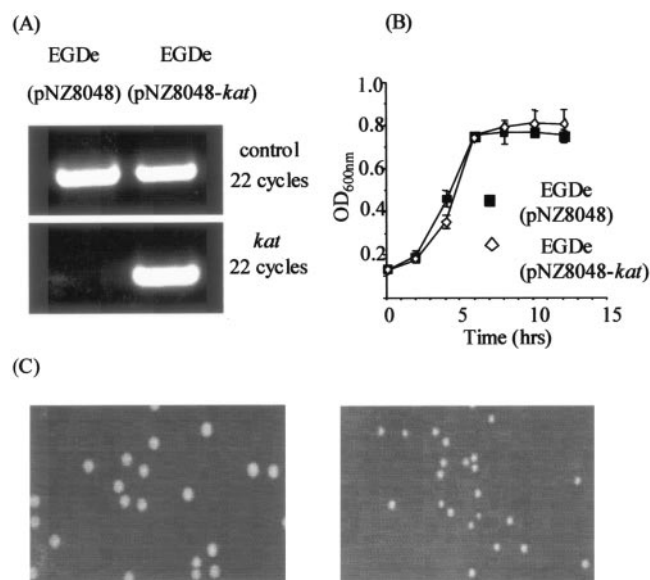


FIG. 5. (A) RT-PCR analysis of gene expression. Control primers were used to ensure that the concentrations of template cDNAs were equal. Samples were removed at various PCR cycles and visualized on agarose gels. (B) Growth of EGDe-NICE(pNZ8048) and EGDe-NICE(pNZ8048-kat) in BHI with 45 µg nisin powder/ml at 37°C. The error bars indicate the standard deviations from the means of triplicate experiments. (C) Growth of EGDe-NICE(pNZ8048) (left panel) and EGDe-NICE(pNZ8048-kat) (right panel) on BHI agar with 45 µg nisin powder/ml.

regulation by PerR. However, allowing two and three mismatches to the consensus sequences resulted in 206 and 1,862 hits, respectively, in the *Listeria* genome. Many sequences could be eliminated based on the position of the PerR box, but the remainder required further bioinformatic analysis. For the purposes of this study we considered only the genes already defined as members of the PerR regulon in *Bacillus*.

(i) **Catalase.** The previously characterized *kat* (30) gene in *Listeria* exhibits 49% identity with *kataA* of *Bacillus*. A PerR box with one mismatch to the consensus sequence was identified 53 bp upstream of the annotated start codon (Fig. 6).

(ii) **Alkyl hydroperoxide reductase.** *ahpC* and *ahpF* encode the two subunits of alkyl hydroperoxide reductase and are cotranscribed in *Bacillus*. In *Listeria* the closest homolog of *ahpC* (32% identity) is *lmo1604* (similar to 2-Cys peroxidase). A PerR box with one mismatch was identified 99 bp upstream of the annotated start codon (Fig. 6). However, there is no adjacent *ahpF* homolog in *Listeria*, and the closest homolog, *trxB* (thioredoxin reductase; 27% identity), is located some distance away at a separate locus. In addition to *ahpF* *Bacillus* also has a *trxB* gene which exhibits 73% homology with *trxB* in *Listeria*. This gene was not found to be PerR regulated in *Bacillus*. However, we included this gene due to the absence of *ahpC* and *ahpF* in *Listeria* and the presence of a PerR box, albeit with four mismatches 320 bp upstream of the annotated start codon (Fig. 6).

(iii) **Ferric uptake regulator.** *fur* in *Listeria* exhibits 76% identity with the corresponding gene in *Bacillus*. A PerR box with a perfect match to the consensus sequence was identified 91 bp upstream of the annotated start codon (Fig. 6).

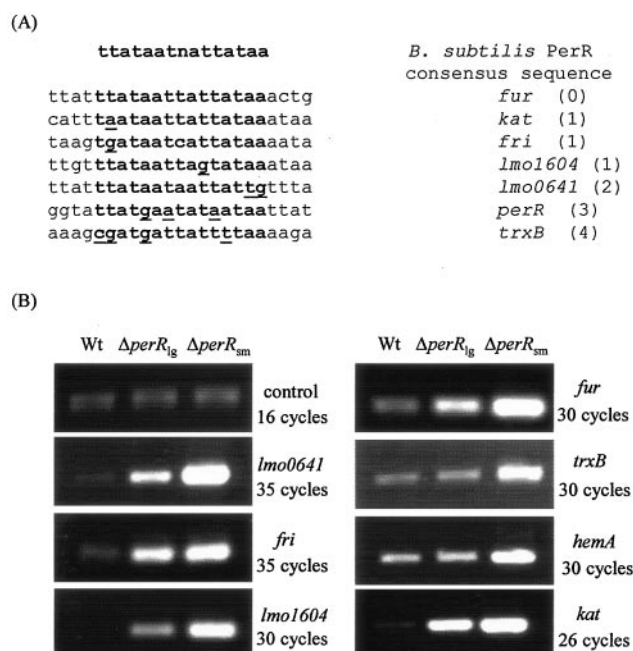


FIG. 6. (A) Alignment of PerR boxes (boldface type) from putative PerR-regulated genes. Boxes were identified by searching the *L. monocytogenes* EGDe genome sequence using the PerR consensus sequence from *B. subtilis*. The numbers in parentheses indicate the numbers of mismatches with the consensus sequence (underlined). (B) RT-PCR analysis was used to confirm PerR regulation of all identified genes from log-phase cultures. Analysis of *kat* expression was included as a control. Primers for a housekeeping gene were used to ensure that the concentrations of template cDNAs were equal. Samples were removed at various PCR cycles and visualized on agarose gels. Wt, wild type.

(iv) **ZoSA.** The closest homolog of the P-type ATPase in *Listeria* is *lmo0641* (54% identity). A PerR box with two mismatches to the consensus sequence was identified 102 bp upstream of the annotated start codon (Fig. 6).

(v) **Metalloregulation DNA-binding stress protein (MrgA).** The closest homolog of the metalloregulation DNA-binding stress protein (MrgA) gene in *Listeria* is the nonheme iron-binding ferritin gene (*fri*). This gene has been shown to be a stress- and starvation-induced gene controlled by sigma-B (37). *mrgA* exhibits 35% identity with *fri*. A PerR box with two mismatches to the consensus sequence was identified 50 bp upstream of the annotated start codon (Fig. 6).

(vi) **HemAXCDBL.** The six-gene HemAXCDBL operon is involved in heme biosynthesis in *Bacillus*. There is a similar operon in *Listeria*, but it contains only five genes. The closest homologue of *hemX* in *Listeria* is located at a separate locus. *hemA* exhibits 47% identity with the corresponding gene in *Listeria*. However, no PerR box was identified upstream of the annotated start codon in *L. monocytogenes*.

The expression of all of the genes described above was analyzed in the two mutant backgrounds. As stated above, the *kat* gene was found to be up-regulated in both the Δ *perR*_{lg} and Δ *perR*_{sm} mutants compared to the wild type, but a greater degree of up-regulation was observed in the Δ *perR*_{sm} mutant. The same phenomenon was also noted for many of the other genes identified as members of the PerR regulon in *L. mono-*

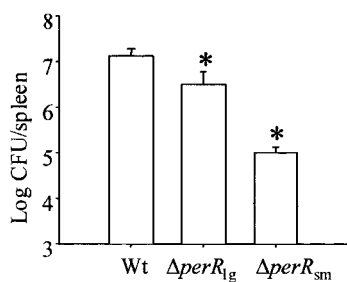


FIG. 7. Effect of deletion of the *perR* gene on survival of *L. monocytogenes* EGDe in vivo. Mice were inoculated intraperitoneally with either the wild type or the mutants, and the numbers of bacteria recovered from the spleen were determined 3 days postinoculation. The error bars indicate the standard deviations from the means ($n = 4$). An asterisk indicates that the mean is significantly different from the wild-type (Wt) mean ($P < 0.05$).

cytogenes. fur, fri, lmo0641, and lmo1604 all displayed expression patterns similar to that of catalase. *trxB* and *hemA* were found to be up-regulated in the $\Delta perR_{sm}$ mutant compared to the wild type. However, the levels of these genes were comparable in the wild-type and $\Delta perR_{lg}$ backgrounds.

Virulence studies. In a previous study we identified a role for PerR in the pathogenesis of *Listeria* (42). However, we did not discriminate between the two colony types, and the *perR* mutant exhibited a 10-fold reduction in splenic numbers compared to the wild type on day 3 of infection. In the current study we compared the virulence of $\Delta perR_{lg}$ and $\Delta perR_{sm}$ mutants and the virulence of the wild type using the intraperitoneal mouse model of infection (Fig. 7). The numbers of bacteria in the spleens of infected mice were determined after 3 days. The levels of both *perR* mutants were lower than those of the wild type in the spleens of infected animals ($P < 0.05$); however, the $\Delta perR_{sm}$ mutant was most significantly affected. The numbers of cells of this mutant recovered from the spleens were 100-fold less than the numbers of wild-type cells. Repeat experiments verified that $\Delta perR_{sm}$ mutants were isolated at significantly lower levels than the wild type and $\Delta perR_{lg}$ mutants (data not shown). Therefore, the secondary mutation resulting in the large colonies is beneficial for *Listeria* in vivo. However, this mutation does not fully restore virulence to wild-type levels, and the rate of appearance of large colonies from the $\Delta perR_{sm}$ mutant in vivo did not differ from the rate encountered in complex broth. These results demonstrate that a loss of PerR activity severely reduces virulence and confirm that the *perR* gene plays a significant role in the virulence of *Listeria*.

DISCUSSION

The ferric uptake repressor (Fur) superfamily is composed of three DNA-binding metalloregulatory proteins that act as repressors of gene expression (Fur, Zur, and PerR). Zur is responsible for zinc uptake and homeostasis (13, 18, 19), whereas Fur is responsible for balancing the intracellular concentration of iron to maintain appropriate levels for normal growth (2). PerR regulates a number of genes which play a critical role in defense against peroxide stress and ROS, and it responds to either Fe or Mn ions (20, 23). In this study we

examined the physiological and genetic consequences of deleting PerR and determined the effects on the ability of *Listeria* to cause disease.

We determined that deletion of *perR* results in two distinct colony types, small colonies ($\Delta perR_{sm}$) and colonies that are similar in size to the wild type yet maintain the *perR* deletion ($\Delta perR_{lg}$) (42). Initial observations only showed that these colonies differ in size, as the texture, hemolytic activity, color, and motility were the same for both colony types. Physiological analyses further separated these two colony types since the $\Delta perR_{sm}$ mutant grew at a slower rate than either the wild type or the $\Delta perR_{lg}$ mutant. Slower growth rates of a *B. subtilis perR* mutant have been reported previously (8, 10, 22). Repeated passaging of the $\Delta perR_{sm}$ mutant gave rise to larger colonies that were similar in size to the wild type ($\Delta perR_{lg}$), which ultimately dominated the culture. Therefore, deletion of the *perR* gene in *Listeria* initially resulted in a strain which produced smaller colonies, had a decreased growth rate, and exhibited increased sensitivity to peroxide stress. Subsequently, larger colonies with increased resistance to peroxide stress appeared, whose growth rate was similar to that of the wild type. Although the genetic change responsible for these larger colonies has not been elucidated (see below), it restored some of the lost fitness to the bacterium. Significantly, previous work with *B. subtilis* also identified two colony sizes following *perR* deletion (10). This study did not identify the precise genetic change that resulted in this phenomenon. However, the authors did determine that the larger colonies lacked catalase activity. Clearly, it is important to comprehend the true nature of the phenotype associated with *perR* deletion in order to understand the significance of this regulon for in vitro and in vivo growth and to inform future transcriptomic and proteomic analyses.

One of the principal physiological differences between the $\Delta perR_{sm}$ and $\Delta perR_{lg}$ mutants is the response to H_2O_2 stress. Transcriptional analysis (RT-PCR and promoter studies) revealed that there was up-regulation of catalase in both backgrounds compared to the wild type. Surprisingly, there were significantly higher levels of the transcript in the $\Delta perR_{sm}$ mutant, even though it was less resistant to H_2O_2 stress than the $\Delta perR_{lg}$ mutant. Therefore, we postulated that creation of a *perR* mutant results in an increase in catalase to levels that are somehow toxic to the cell, resulting in smaller colonies and an inability to resist peroxide stress. Indeed, in support of this hypothesis, we determined that significant overexpression of catalase in wild-type *L. monocytogenes* resulted in a small-colony phenotype and decreased resistance to H_2O_2 . It is interesting that many previous reports have suggested that deletion of PerR in gram-positive bacteria results in increased resistance to H_2O_2 (6, 8, 24, 28, 43). We concluded here that this is not the true phenotype associated with a PerR mutation in *L. monocytogenes* and that care must be taken when $\Delta perR$ colonies are selected for further study. Interestingly, deletion of PerR in *Synechocystis* sp. resulted in a strain which responded to H_2O_2 stress in a manner similar to the wild type (31). The increased H_2O_2 sensitivity of a $\Delta perR_{sm}$ mutant may be due to the higher levels of Fur present in this mutant. Fur is required to derepress iron uptake genes under iron limitation conditions. Iron is cofactor for many important enzymes in the cell, including catalase. The increased level of Fur may

prevent derepression of iron uptake genes, resulting in iron starvation. Any remaining iron within the cell is incorporated only into essential proteins. This may hinder the ability of the catalase enzyme to work efficiently.

It is clear from our work that a subsequent genetic alteration gives rise to large colonies ($\Delta perR_{lg}$) from the small-colony background. The large-colony mutants exhibit a relative reduction in levels of *kat* expression, alleviation of the growth defect, and increased resistance to H_2O_2 . We speculated that this phenomenon might result from secondary mutation of the *kat* locus. Sequence analysis revealed that no base changes had occurred in the *kat* promoter or structural gene in either $\Delta perR_{sm}$ or $\Delta perR_{lg}$ mutants, suggesting that the genetic events that give rise to increased fitness in a $\Delta perR_{lg}$ mutant reside outside the *kat* locus, perhaps in another, as-yet-undefined regulator of catalase expression.

Indeed, increased transcription in the $\Delta perR_{sm}$ mutant background appears to extend beyond the *kat* locus to affect other PerR-regulated genes. We used a bioinformatic approach (3) to identify a number of genes as putative members of the PerR regulon and examined their expression in both mutant backgrounds. The identification of PerR-regulated genes in *L. monocytogenes* adds to the recent report of a PerR-regulated iron-binding protein (Fri) in this pathogen (37). The PerR-regulated genes identified in this study are also part of the PerR regulon in *B. subtilis* and include *kat* and *ahpCF* (defense against peroxide stress), *mrgA* (protection of DNA), *hemAXCDBL* (heme biosynthesis), *zosA* (zinc uptake), and *fur* (regulation of iron homeostasis) (19, 23). It was significant that the expression of these genes in the two $\Delta perR$ mutants followed a pattern similar to that displayed by *kat*. Most of the genes were up-regulated in both backgrounds, and a greater increase in expression was observed in the $\Delta perR_{sm}$ mutant. It is interesting that the bioinformatic search also identified a number of putative regulators as likely candidates for regulation by PerR. Strong matches with the consensus sequence were identified upstream of the start codons of lmo0416, lmo0106, lmo2146, and lmo2165. It is possible that in the absence of PerR one of these regulators results in the altered gene expression observed in a $\Delta perR_{lg}$ mutant. However, this requires further investigation.

Studies with *S. aureus* have shown that PerR is an essential regulator that is required for virulence in a murine subcutaneous skin abscess model of infection (24). We previously demonstrated a role for PerR in the infectious cycle of *Listeria* using, in hindsight, what we now know to have been a $\Delta perR_{lg}$ mutant (42). That study demonstrated that a moderate (10-fold) reduction in virulence potential was associated with the *perR* deletion. In the current study the $\Delta perR_{sm}$ mutant, which we believe represents the true $\Delta perR$ phenotype, exhibited a significant and substantial reduction in virulence compared to both the wild type (100-fold reduction) and the $\Delta perR_{lg}$ mutant (10-fold reduction). This shows that environmental adaptation through PerR is essential for complete virulence in *L. monocytogenes* and that the true phenotype may be under-represented in clones that have developed a secondary mutation ($\Delta perR_{lg}$). It is likely that the $\Delta perR_{sm}$ mutant examined in this study differs from the general small-colony variants that have been isolated from infections caused by a variety of gram-negative and gram-positive organisms (9, 44, 45, 48, 49). These small-colony variants actually have a greater capacity to persist

intracellularly than the wild type, whereas the $\Delta perR_{sm}$ mutant has a greatly reduced virulence potential.

In conclusion, deletion of the gene encoding the PerR repressor results in increased expression of its regulon, probably at such a level that it decreases the fitness of the cell, resulting in a slower growth rate and increased sensitivity to peroxide stress. This is, at least in part, due to the increase in catalase activity in the cell. This imposes selection pressure on the cell, which results in a secondary, unidentified mutation, which results in down-regulation of the PerR regulon to a level which restores fitness but is still greater than the wild-type level of expression. This is an important finding that should influence the design of future experiments to examine the nature of PerR-dependent gene regulation in *Listeria* spp. and related organisms.

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