

Involvement of the Mannose Phosphotransferase System of *Lactobacillus plantarum* WCFS1 in Peroxide Stress Tolerance[∇]

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A *Lactobacillus plantarum* strain with a deletion in the gene *rpoN*, encoding the alternative sigma factor 54 (σ^{54}), displayed a 100-fold-higher sensitivity to peroxide than its parental strain. This feature could be due to σ^{54} -dependent regulation of genes involved in the peroxide stress response. However, transcriptome analyses of the wild type and the mutant strain during peroxide exposure did not support such a role for σ^{54} . Subsequent experiments revealed that the impaired expression of the mannose phosphotransferase system (PTS) operon in the *rpoN* mutant caused the observed increased peroxide sensitivity.

The lactic acid bacterium *Lactobacillus plantarum* is encountered in many dairy, meat, and plant fermentations. Furthermore, it is frequently encountered in the human gastrointestinal tract (1), and some strains are marketed as probiotics (6). During exponential growth, *L. plantarum* converts glucose almost completely to lactate (8). However, under aerobic conditions, a pathway involving lactate dehydrogenase, pyruvate oxidase, and acetate kinase enzymes can convert lactate to acetate and produces one ATP (10, 11, 15). This pathway produces hydrogen peroxide (H₂O₂) and carbon dioxide (CO₂) as side products, and accumulation of peroxide ultimately leads to aerobic growth arrest (3). Together with superoxide (·O₂⁻) and hydroxyl radicals (·OH), hydrogen peroxide belongs to a group of compounds known as reactive oxygen species (ROS). Hydrogen peroxide is relatively inert toward organic compounds (5), but it reacts readily with metal ions like Fe²⁺ to yield hydroxyl radicals (Fenton's reaction) that damage DNA, proteins, and membranes (9). Analyses of the genome sequence of *Lactobacillus plantarum* WCFS1 revealed the presence of a sophisticated defense system against hydrogen peroxide, which includes a putative glutathione peroxidase-encoding gene (*gpo*) (14). An *in silico* regulatory network prediction for the alternative sigma factor 54 (σ^{54}) suggested σ^{54} -dependent expression of *gpo* (19), postulating a role for σ^{54} in the oxidative stress response of *L. plantarum*.

In this article, we describe the increased peroxide sensitivity of an *L. plantarum* *rpoN* mutant (*rpoN::cat*; lacking a functional σ^{54}) compared to its parental strain. Subsequent

experiments, including transcriptome analyses and oxidative stress tolerance measurement in mannose phosphotransferase system (PTS) deletion strains, revealed that the previously reported impaired expression of the mannose PTS operon in the *rpoN* mutant (15), rather than the postulated σ^{54} -dependent expression of *gpo*, is responsible for the observed increased peroxide sensitivity.

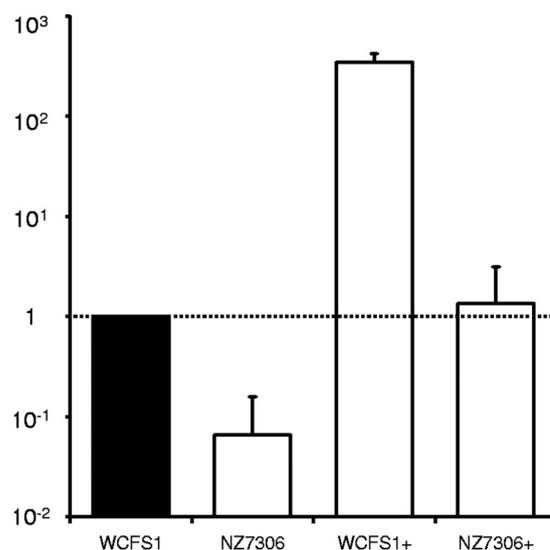


FIG. 1. Relative hydrogen peroxide (40 mM) survival of *L. plantarum* WCFS1 (wild type) and NZ7306 (*rpoN::cat*) after 30 min. Survival was measured with (+) or without 30 min of adaptation to a sublethal level of hydrogen peroxide (3.5 mM). The survival of the wild-type strain (set at 1.0) was used to normalize the relative survival observed in the same strain after adaptation to sublethal levels of hydrogen peroxide or the relative survival observed in the mutant strain. The actual viability counts of the wild-type strain reduced from $(2.75 \pm 1.77) \times 10^8$ to $(4.15 \pm 0.47) \times 10^4$ to underline the lethality of the dosage used. Results presented are the average relative survival rates of three independent experiments, including the standard deviation between experiments.

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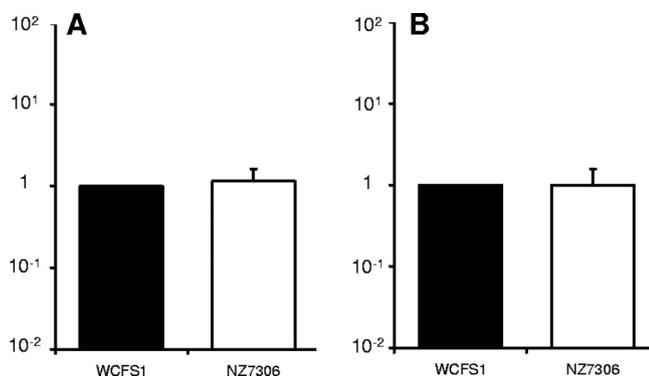


FIG. 2. (A) Relative survival of *L. plantarum* WCFS1 (wild type) and NZ7306 (*rpoN::cat*) in MRS medium after 30 min of heat stress exposure (60°C). (B) Relative survival of *L. plantarum* WCFS1 (wild type) and NZ7306 (*rpoN::cat*) after 5 min of exposure to UV light (302 nM). Cells were grown to an optical density of 1.0 and immediately incubated at 60°C or harvested and resuspended in 50 mM KPO₄ buffer supplemented with 2% glucose followed by UV light exposure. The survival of the wild-type strain (set at 1.0) was used to normalize the relative survival of the mutant strain after heat or UV stress exposure. The actual viability reductions observed in the wild-type strain under these conditions were from $(1.09 \pm 0.16) \times 10^8$ (prior to stress exposure) to $(5.90 \pm 2.69) \times 10^4$ and $(5.30 \pm 0.42) \times 10^4$, respectively. Results presented are the average relative survival rates observed in two independent experiments, including the standard deviation between experiments.

Deletion of the *rpoN* gene, encoding σ^{54} , leads to peroxide sensitivity. To investigate the possible role of σ^{54} in peroxide stress survival, wild-type and *rpoN* mutant cells were grown in MRS until an optical density at 600 nm (OD₆₀₀) of 1.0 was reached, after which hydrogen peroxide was added to a final concentration of 40 mM. Samples were taken after 30 min and immediately diluted in MRS, and CFU were enumerated by plating appropriate dilutions. The 40 mM dosage of hydrogen peroxide required for lethality in *L. plantarum* appears to be relatively high compared with dosages reported to be lethal for other species, like *Lactococcus lactis* and *Streptococcus pyogenes* (13, 16). However, the culture density at which the peroxide stress was applied was significantly lower in those studies than in the work presented here, which may affect the actual concentration experienced by individual cells. In addition, the complexity of MRS medium may add to this difference, since certain compounds present in this medium may scavenge the oxygen radicals derived from hydrogen peroxide, thereby reducing the effective concentration of this stress agent (17). Alternatively, this difference could be explained by the relatively large repertoire of *L. plantarum* functions that are potentially involved in oxidative stress tolerance, including NADH oxidases, glutathione (GSH) reductases, a GSH peroxidase, NADH peroxidases, and thioredoxins (14).

Due to peroxide treatment, the relative viable count of the wild-type culture appeared to be reduced by approximately 3 orders of magnitude after 30 min, whereas that of NZ7306 (*rpoN::cat*) was reduced at least 100-fold more (Fig. 1). This increased sensitivity of NZ7306 could be caused by σ^{54} -dependent regulation of genes involved in the oxidative stress-specific response of *L. plantarum* or genes involved in the general stress response in this organism. To evaluate the latter possibility,

TABLE 1. *L. plantarum* strains used in this study^a

Strain	Relevant features	Reference
WCFS1	Wild type, human isolate	14
NZ7306	<i>rpoN::cat</i> , replacement of the <i>rpoN</i> gene with a chloramphenicol resistance cassette	19
NZ7307	<i>manR::cat</i> , replacement of the <i>manR</i> gene with a chloramphenicol resistance cassette	19
NZ7308	<i>manIIC::pNZ7350</i> , disruption of the <i>manIIC</i> gene by single-crossover plasmid integration	19

^a Strains were grown anaerobically in MRS (4) at 30°C.

the relative capacities of the wild type and its *rpoN* mutant derivative to survive lethal levels of UV and heat stress were determined, and they did not appear to differ significantly (Fig. 2). Therefore, the reduced stress tolerance observed in NZ7306 (*rpoN::cat*) appears to be specific for peroxide stress, which would support a regulatory role of σ^{54} in the control of a candidate peroxide stress tolerance factor, such as glutathione peroxidase.

The role of σ^{54} in survival of acute peroxide stress in *L. plantarum* could relate to σ^{54} -dependent adaptation to peroxide stress conditions. To compare peroxide stress adaptation capacity between the wild type and NZ7306, *L. plantarum* cultures were pretreated with a sublethal peroxide concentration (3.5 mM for 30 min) prior to addition of a lethal peroxide dose. Addition of 3.5 mM hydrogen peroxide resulted in a temporal growth stagnation for approximately 2 h, after which growth resumed, indicating that the concentration of hydrogen peroxide was indeed sublethal. In both strains, adaptation induced an approximate 100-fold-improved relative survival (Fig. 1), suggesting that the adaptation capacity is not affected by the *rpoN* mutation.

Genome-wide analysis of strains WCFS1 and NZ7306 with and without peroxide. To evaluate the possible involvement of σ^{54} in transcription of the glutathione peroxidase gene (*gpo*) or other genes required for peroxide stress survival, full-genome

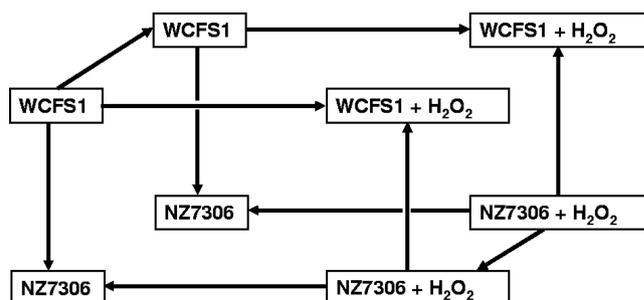


FIG. 3. hybridization scheme of the transcriptome analyses. Two conditions were tested: the condition “peroxide stress exposure” and the condition “deleted *rpoN* gene,” resulting in 4 samples: “WCFS1 (wild type),” “NZ7306 (*rpoN::cat*),” “WCFS1 + H₂O₂,” and “NZ7306 + H₂O₂.” Samples were hybridized against a sample with only one variation, resulting in a total of 4 hybridizations (wild type versus mutant, “wild type versus wild type + peroxide,” “mutant versus mutant + peroxide,” and “wild type + peroxide versus mutant + peroxide”). The experiment was performed in duplicate, and in order to determine the variation between the duplicates, additional hybridizations were performed: “WCFS1 versus WCFS1” and “NZ7306 + H₂O₂ versus NZ7306 + H₂O₂.” Each arrow represents a single hybridization; samples at the base of the arrow were Cy3 labeled, and samples at the arrowhead were Cy5 labeled. Transcriptome data were submitted to the gene expression omnibus (GEO).

TABLE 2. *L. plantarum* genes that are differentially affected by peroxide treatment (3.5 mM) in the wild type compared to the *rpoN* mutant NZ7306 (interaction effect)^a

Description and ORF	Name	Product	Ratio (log ²)	P value (FDR)	B value
Cell envelope					
lp_0618	<i>lp_0618</i>	Cell surface hydrolase, membrane bound (putative)	0.68	1.43E-02	2.14
lp_1185	<i>cpsII</i>	Polysaccharide polymerase	0.75	1.70E-02	1.57
Central intermediary metabolism					
lp_1686	<i>lp_1686</i>	Acyl-coenzyme A thioester hydrolase (putative)	-0.74	1.40E-02	3.16
Fatty acid and phospholipid metabolism					
lp_1670	<i>fabZ1</i>	(3R)-Hydroxymyristoyl-[acyl carrier protein] dehydratase	-1.01	1.40E-02	3.45
lp_1675	<i>fabF</i>	3-Oxoacyl-[acyl carrier protein] synthase II	-0.89	1.43E-02	2.49
lp_1682	<i>lp_1682</i>	Phosphopantetheinyltransferase	-0.78	1.43E-02	2.16
Hypothetical protein					
lp_0199	<i>lp_0199</i>	Unknown	-0.57	1.43E-02	2.07
lp_0291	<i>lp_0291</i>	Oxidoreductase	-0.61	1.84E-02	1.27
lp_1098	<i>lp_1098</i>	Unknown	-0.69	1.43E-02	2.49
lp_1684	<i>lp_1684</i>	Integral membrane protein	-0.84	1.40E-02	3.79
lp_3256	<i>lp_3256</i>	DegV family protein	-0.73	1.92E-02	1.09
lp_3353	<i>lp_3353</i>	Unknown	-0.58	1.59E-02	1.81
Other categories					
lp_1687	<i>lp_1687</i>	GTPase	-0.59	1.88E-02	1.15
Purines, pyrimidines, nucleosides and nucleotides					
lp_0693	<i>nrdE</i>	Ribonucleoside diphosphate reductase, alpha chain	1.01	8.88E-03	5.04
Regulatory function					
lp_1685	<i>lp_1685</i>	Transcription regulator	-0.78	1.40E-02	3.72
Transcription					
lp_0520	<i>rhe1</i>	ATP-dependent RNA helicase	0.78	1.43E-02	2.01
Transport and binding proteins					
lp_0180	<i>msmK1</i>	Multiple sugar ABC transporter, ATP-binding protein	0.89	1.43E-02	2.4
lp_0265	<i>pts5ABC</i>	PTS system, trehalose-specific IIBC component	0.70	1.43E-02	2.15
lp_0315	<i>potD</i>	Spermidine/putrescine ABC transporter, substrate binding protein	0.46	1.90E-02	1.13
lp_0344	<i>tagH</i>	Teichoic acid ABC transporter, ATP-binding protein	0.51	1.86E-02	1.2

^a Positive log² (ratio) values indicate that the effect of peroxide treatment leads to a higher expression in the wild type than in the mutant. Statistical analyses were performed with the R software program (<http://www.r-project.org/>) using the linear models for the microarray data library limma (18) as described previously (20). The *P* values are based on the false discovery rate (FDR); the *B* value is the log odd for differential expression. ORF, open reading frame.

transcriptome analyses were performed as described previously (20). To this end, wild-type strain WCFS1 and its *rpoN* mutant derivative (Table 1) were grown to mid-exponential phase (OD₆₀₀ = 1.0) and treated with 3.5 mM hydrogen peroxide for 30 min. Subsequently, cells were harvested and RNA was isolated for transcriptome profiling (Fig. 3). This profiling revealed only a small difference between the peroxide response of the wild type and that of NZ7306 (*rpoN::cat*), exemplified by the relatively small list of genes displaying an interaction effect, i.e., a significant differential response to peroxide between NZ7306 and the wild type (Table 2). The GSH peroxidase gene was expressed at a higher level in both strains during peroxide stress (data not shown), suggesting that the regulation of this gene does not depend on σ^{54} . The ratios of the genes displaying an interaction effect are low, and therefore, the transcriptome analyses failed to disclose direct clues that could explain the increased peroxide sensitivity observed in the σ^{54} mutant.

Peroxide sensitivity of strains lacking a mannose PTS. The transcriptome analyses showed no significant difference in peroxide exposure between the wild type and NZ7306 (*rpoN::cat*), raising the question of whether the peroxide sensitivity of NZ7306 is caused by the reported impaired expression of the mannose PTS operon in this strain (19). To investigate the putative role of the mannose PTS in peroxide tolerance, the hydrogen peroxide sensitivities of two strains, NZ7307 and NZ7308, lacking expression of a functional mannose PTS were tested. NZ7307 is mutated in the mannose operon regulator gene *manR*, whereas NZ7308 harbors a mutation in the transferase-encoding gene *manIIC* (Fig. 4; Table 1 [19]). When these mannose PTS mutants were treated with a lethal dose of hydrogen peroxide, the viable cell counts showed a 1- to 2-order-of-magnitude-higher reduction than the wild type (Fig. 5), which parallels the reduction observed for the *rpoN* mutant, NZ7306 (Fig. 1). Since NZ7306, NZ7307, and NZ7308 share the lack of expression of a functional mannose PTS, these

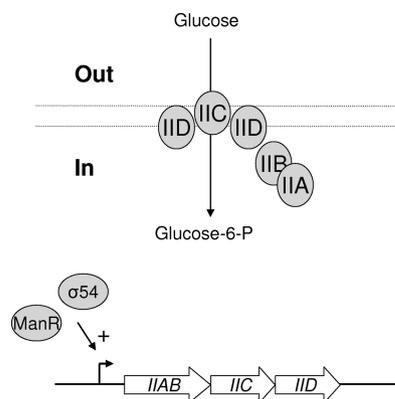


FIG. 4. Schematic representation of regulation of the mannose PTS in *L. plantarum*. The mannose operon is regulated by sigma 54 (encoded by the gene *rpoN*) in concert with the transcriptional regulator ManR. The mannose operon codes for the mannose PTS system, and its product consists of an IIA protein, involved in phosphate transfer from the histidine phosphocarrier protein to the transporter (IIC) in the membrane. The membrane protein IID is involved in guidance of phosphorylated IIA to IIC (7).

results seem to point at a direct relation between the presence of a functional mannose PTS and peroxide tolerance in *L. plantarum*.

The mannose PTS is a major glucose-transporting PTS in various lactic acid bacteria (2), and deletion of this transporter leads to reduced growth in *L. plantarum* (15), which is probably due to reduced glucose uptake capacity. Reduced glucose uptake in cells lacking a functional mannose PTS could lead to a reduction in the energy generation rate, which may cause increased peroxide sensitivity. However, this “lack-of-energy” explanation would also predict an increased sensitivity to other forms of stress (e.g., UV, heat, etc.), which could not be experimentally confirmed. Comparative analysis of mannose PTSs suggests a relatively late evolutionary origin of this transport system (21) and phylogenetic profiling placed the *Escherichia coli* and *L. plantarum* mannose PTSs in the same highly conserved group of mannose transporters (21). The mannose PTS homolog in *E. coli* is highly resistant to oxidizing agents (12), and the close relationship between the systems suggests a similar robustness of the *L. plantarum* system. This suggests the maintenance of the glucose import function of the mannose PTS during peroxide exposure, while the alternative transporters that are used for glucose import in the mutant strains (NZ7306, NZ7307, and NZ7308) are inactivated under these conditions. Consequently, the cells that lack a mannose PTS will have major problems in energy generation processes that are required to launch an appropriate peroxide-induced stress response, ultimately leading to increased peroxide sensitivity. Overall, our results indicate a role of the mannose PTS in oxidative stress tolerance in *L. plantarum* and corroborate the previously observed resistance to oxidizing agents of this family of transport systems.

Oxidative stress is an industrially relevant stress condition, which may be encountered during processing or as a consequence of hydrogen peroxide production as a side product of carbohydrate fermentation. Therefore, our findings imply that specific culture conditions that induce expression of the man-

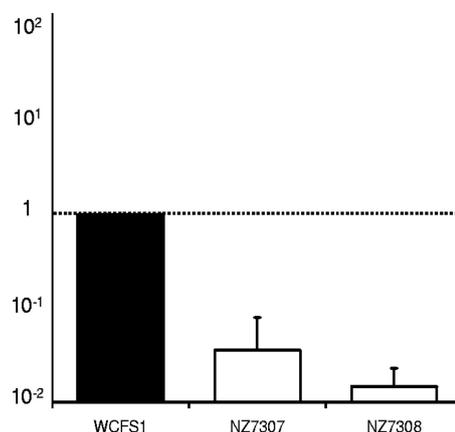


FIG. 5. Relative hydrogen peroxide (40 mM) survival of *L. plantarum* WCFS1 (wild type) and the mutant strains NZ7307 ($\Delta manR$) and NZ7308 ($\Delta manIIC$). The absolute survival reduction rates observed for the wild-type strain are provided in the legend of Fig. 1. Results presented are the average relative survival rates compared to the wild-type rate from two independent experiments, including the standard deviation between experiments.

nose PTS (i.e., growth on specific carbon sources like glucose or mannose [15]) allow the production of bacterial cells displaying increased oxidative stress tolerance, which is relevant for starter-culture production and fermentation industries.

Microarray data accession numbers. The microarray design was submitted to the Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under GEO accession number GPL6368. Primary transcriptome data were submitted to GEO under accession number GSE-11351.

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