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BACTERIAL FUNGAL AND VIRUS MOLECULAR BIOLOGY - RESEARCH PAPER

# Characterization of genes responsive to osmotic and oxidative stresses of the sugarcane bacterial pathogen *Leifsonia xyli* subsp. *xyli*

Raphael S. C. A. Faria[1] · Mariana C. Cia[1] · Claudia B. Monteiro-Vitorello[2] · Ricardo A. Azevedo[2] · Luis Eduardo A. Camargo[1]

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## Abstract

*Leifsonia xyli* subsp. *xyli* (Lxx) colonizes the xylem vessels of sugarcane, a plant niche where microorganisms are highly exposed to oxidative and osmotic stresses. This study performed an *in silico* analysis of the genome of Lxx and characterized 16 genes related to the detoxification of oxidative species (peroxidases, $O_2^-$ dismutases, and methionine reductases) and to the production and transport of osmolytes and analyzed their expression *in vitro* after 30, 60, and 120 min of exposure to $H_2O_2$ or PEG. The PAGE activity of superoxide dismutase (Mn-SOD as confirmed by inhibition tests) and of catalase (CAT) and the accumulation of trehalose were also assessed. Exposure to $H_2O_2$ increased the expression of most oxidative-responsive genes and decreased the expression of those related to osmotic responses, whereas the opposite occurred after exposure to PEG. The isoform profiles of CAT and Mn-SOD shifted in response to $H_2O_2$ but not to PEG and Lxx cells accumulated more trehalose over time after exposure to PEG compared with non-exposed cells. The experimental results validated the *in silico* analysis and indicated that this obligate endophytic parasite has multiple and functional mechanisms to combat the stresses imposed by its host.

**Keywords** Ratoon stunt disease · Gene expression · Reactive oxygen species · Osmolytes

## Introduction

To establish a successful parasitic relationship, pathogens must first cope with stresses of diverse nature imposed by their host environments such as nutrient and oxygen deprivation and exposure to oxidative and osmostressing agents [1, 2]. Like other living organisms, bacteria developed several mechanisms to protect from, withstand, or repair the damages inflicted by these stressing agents. Among them, the ones involved in the combat of ROS and osmostressing agents are the most studied [1, 2]. Enzymes involved in the detoxification of hydrogen peroxide ($H_2O_2$) and superoxide anion ($O_2^-$) and in the repair of oxidized amino acid residues, mainly cysteine and methionine, are examples related to the first type of stress, while the production or transport of osmolytes such as the non-reducing sugar trehalose and glycine betaine ABC transporters are examples of the second.

The actinobacterium *Leifsonia xyli* subsp. *xyli* (Lxx) belongs to a restricted group of plant pathogenic and fastidious organisms that inhabit the xylem vessels of their hosts. It is considered an obligate endophyte of sugarcane because it has no free-living habit and its only natural host described so far is *Saccharum* spp. [3, 4]. Plant genotype and age are factors that affect bacterial titers in the plant [5] which ultimately affect plant growth to such an extent that highly colonized plants of susceptible varieties present visible shorter internodes and reduced stem diameter. As these symptoms are more evident in ratoon plants, i.e., plants that sprout from the stubble after harvesting of the aboveground portion, the disease is named "ratoon stunt". Growth reduction is believed to result from restricted water movement inside the plant due to the clogging of the xylem by a matrix possibly produced by the plant to

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✉ Luis Eduardo A. Camargo
leacamar@usp.br

[1] Department of Plant Pathology and Nematology, Luiz de Queiroz Agricultural School, University of São Paulo, Piracicaba, SP 13418-900, Brazil

[2] Department of Genetics, Luiz de Queiroz Agricultural School, University of São Paulo, Piracicaba, SP 13418-900, Brazil

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restrict the spread of the pathogen within its vascular system [6]. Symptoms of ratoon stunt are more severe when sugarcane is grown under conditions of water stress [7], thus aggravating the availability of water. In addition to the clogging effect, recent data indicate that *Leifsonia x.* subsp. *xyli* down-regulates the cell-division cycle in young plants with no apparent symptoms of water restriction, suggesting that an additional factor possibly of bacterial origin may also operate to affect plant growth [8]. Besides interfering in the cell cycle, Lxx also alters the hormonal balance of sugarcane, negatively affects various photosynthetic parameters, and modulates the activities of peroxidase, catalase, and superoxide dismutase in concert with an increased production of superoxide anion radical ($O^{2-}$) [8, 9].

*Leifsonia x.* subsp. *xyli* has a peculiar genome as approximately 13% of its genes are predicted to be non-functional, a proportion significantly higher relative to other bacterial plant pathogens, including *Xylella fastidosa* 9a5c, another xylem-dwelling pathogen but with a larger host range whose genome has only 2.9% of pseudogenes [10]. Thus, the accumulation of pseudogenes in the genome of Lxx probably contributed to restrict its host range [10]. Also noteworthy is the finding that only 105 genes are predicted to play any possible role in pathogenicity, of which the largest class comprises 36 genes related to adaptation to stressing conditions, generally categorized as toxin-related efflux-pumps and oxidative protectant molecules [10]. However, more detailed analyses of these genes at the bioinformatic and functional levels have not been performed to date.

This study conducted a bioinformatic search in the genome sequence of *Leifsonia x.* subsp. *xyli* for genes coding for enzymes and other compounds known to be directly involved in the protection of prokaryotic cells against oxidative and osmostressing agents and quantified the levels of cell lipid peroxidation and the expression of a set of these genes *in vitro* after exposure of bacteria to hydrogen peroxide ($H_2O_2$) and to polyethylene glycol (PEG6000). Additionally, we characterized the activity in non-denaturing PAGE of superoxide dismutase and of catalase and quantified the production of trehalose in the presence of these compounds.

## Material and methods

### Bacterial strain and culturing

The CTCB07 strain of *Leifsonia xyli* subsp. *xyli* (Lxx) was used in all experiments. This strain is deposited in the collection of the Instituto Biológico (Campinas, São Paulo, Brazil) under the number IBSBF 1853, and the access number of its genome sequence in the GenBank is NC_006087. The bacteria were cultured in MSC medium [10, 11] at 28 °C under agitation (150 rpm) in an orbital shaker.

### *In silico* analysis of osmotic and oxidative stress-related genes, and primer design and gene amplification

An in silico analysis of the annotated genome sequence of Lxx CTCB07 was performed in the genome database of the Laboratory of Bioinformatics of the University of Campinas (http://bioinfo03.ibi.unicamp.br/lbi/index.html). Keyword searches were performed for non-truncated genes involved in bacterial defense against oxidative and osmotic stresses. In the first case, emphasis was given to genes coding for haem and non-haem peroxidases described in the *Actinomycetales* [12], superoxide dismutases, and methionine sulfoxide reductases involved in the repair of oxidized methionine residues [13]. The search for osmoprotective genes focused on the biosynthesis of the osmolytes trehalose and N-acetylglutaminylglutamine amide and glycine betaine osmolyte transporters. The identities of the genes were confirmed by blastn and blastx searches [14] against the non-redundant nucleotide and protein databases of GenBank.

Primers were designed with the *Beacon Designer* software (http://www.premierbiosoft.com/qOligo/Oligo.jsp?PID=1) with the following parameters: (a) amplicon size from 100 to 150 bp, (b) primer size between 17 and 23 bp, (c) annealing temperature between 57 and 69 °C. Primers with potential hairpin, self-dimer, and cross-dimer structures were excluded. Amplification reactions for primer testing consisted of 12.5 μL of 2× master mix buffer (Promega), 0.3 μM of each primer, 50 ng of DNA, and nuclease-free water (Ambion, Netherlands) in a final volume of 25 μL. The amplification program consisted of an initial cycle at 95 °C for 5 min followed by 40 cycles of 10 s at 95 °C and 30 s at 54–68 °C (depending on the primers).

### Quantification of lipid peroxidation in Lxx exposed to $H_2O_2$ and PEG 6000

An experiment with 4 treatments was designed to determine if exposure of Lxx to $H_2O_2$ or PEG 6000 resulted in stress as assessed by the level of cell lipid peroxidation. Treatments consisted of exposing Lxx to 30 mM of $H_2O_2$ or to 7% (w/v) of PEG 6000 for 60 min. The controls consisted of unexposed cultures analyzed immediately before (control 0 or C) or 60 min (control 60 or C60′) after the addition of the compounds to the other treatments. The medium (800 mL) was inoculated with 8 mL (1% v/v) of a start bacterial culture of $O.D._{(600nm)} = 0.7$ (approximately 8 days of culturing) and then 50-mL aliquots were transferred into sixteen 125-mL Erlenmeyer flasks. The cultures were incubated for 7 days until they reached an approximate O.D. of 0.7 when the stressing agents were added. Bacterial cells were precipitated, and lipid peroxidation was determined through the quantification of metabolites reactive to 2-thiobarbituric acid (TBA),

mainly malondialdehyde (MDA) [15, 16]. The concentration of MDA was expressed as nmol/mL of solution. Four replicate assays were conducted.

## Gene expression after exposure to $H_2O_2$ and PEG 6000

Sixteen 50-mL Lxx cultures were established in 125-mL flasks as described and were incubated at 28 °C for 7 days under shaking at 150 rpm until they reached an $O.D._{(600nm)}$ of approximately 0.7. Then, 170 μL of 30% $H_2O_2$ (Merck) were added to twelve flasks, resulting in a final concentration of 30 mM. Bacterial cells were precipitated at 30, 60, and 120 min after exposure to hydrogen peroxide by centrifugation for 10 min at 12,000×*g*, frozen in liquid nitrogen and stored at − 80 °C. Cells of the four remaining flasks, to which $H_2O_2$ was not added, were harvested just before the addition of the compound (time zero) and served as the experimental control. A second experiment was carried out following the same procedure, except that the cultures were exposed to 7% (w/v) of PEG 6000. In both trials, four replicate assays were conducted.

Total RNA was extracted according to Bowtell and Sambrook (2003) [17], with the addition of 10 mg/mL lysozyme in the extraction buffer and used in a reverse transcription reaction using the GoScript™ Reverse Transcription System (Promega) to synthesize cDNA. Real-time PCR was carried out in a 7500 Fast thermal cycler (Applied Biosystems) using the GoTaq® Real-Time qPCR and RT-qPCR Systems for Dye-Based Detection (Promega) with one technical replicate for each sample. The amplification conditions followed the ones described before. A set of seven genes were tested as normalizers (Table 1) and their expression levels were analyzed with RefFinder to evaluate their expression stability. Genes with the smallest geometric means were considered more stable and used as normalizers. Gene expression data were analyzed with the Relative Expression Software Tool- REST software [18]. The reaction efficiency was estimated using LinReg PCR [19].

## Catalase and superoxide dismutase PAGE activity assays

The experimental design was the same as described for the gene expression analysis. Liquid cultures were centrifuged at 12,000×*g* for 15 min at 4 °C and the cell pellets were macerated with liquid nitrogen. The extracts were homogenized in 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 3 mM DL-dithiothreitol (DTT), and 5% (w/w) polyvinylpolypyrrolidone (PVPP) [20]. The homogenate was centrifuged at 12,000×*g* for 30 min and the supernatant was stored in separate aliquots at 80 °C for further analyses. The protein concentration in all samples was determined using bovine serum albumin as a standard [21].

Preparation and running conditions of the non-denaturing polyacrylamide gel electrophoresis (PAGE) were carried out as described by Peters et al. (2014) [22]. Superoxide dismutase (SOD, EC 1.15.1.1) activity staining was carried out as reported by Azevedo et al. (1998) [23]. After non-denaturing PAGE separation, the gel was rinsed in deionized water and incubated in the dark in 50 mM potassium phosphate buffer (pH 7.8) containing 1 mM EDTA, 0.05 mM riboflavin, 0.1 mM nitroblue tetrazolium, and 0.3% N,N,N′,N′-tetramethylethyllenediamine. One unit of bovine liver SOD (Sigma, St. Louis, USA) was used as a positive control. After 30 min, the gels were rinsed with deionized water and then illuminated in water until the development of achromatic bands of SOD activity on a purple-stained gel. The PAGE activity of catalase (CAT, EC 1.11.1.6) was determined as reported by Peters et al. (2014) [22]. Gels were incubated in 0.003% $H_2O_2$ for 10 min and developed in a 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN_6)$ solution for 10 min. One unit of bovine liver CAT (Sigma, St. Louis, USA) was used as a positive control.

SOD isoforms were determined based on their sensitivity to 2 mM potassium cyanide (KCN) and 5 mM $H_2O_2$ according to Guelfi et al. (2005) [24]. Three PAGE gels were run as described but using 200 μg of protein instead corresponding to the control (0)-, 60-, and 120-min treatments. These treatments were chosen so that all isoforms were represented based on previous unreported experiments. The characterization was done by dividing the gel vertically into three parts. The first part was maintained at 4 °C in 100 mM potassium phosphate buffer pH 7.8 being the control where all isoforms were expected to be present; the second was immersed in 100 ml of the same buffer containing 2 mM KCN and 1 mM EDTA; the third was immersed in 100 ml of buffer containing 5 mM $H_2O_2$ and 1 mM EDTA. The gels were kept in the dark during the treatments. After 20 min in these solutions, the gels were developed with nitroblue tetrazolium and riboflavin. At the end of the development, the presence or absence of bands in the control and in the treatments with KCN and $H_2O_2$ was analyzed. The isoforms were then classified as Mn-SOD, Fe-SOD, or Cu/Zn-SOD, where Mn-SOD is not inhibited in any of the treatments (KCN and $H_2O_2$), Fe-SOD is not inhibited by KCN but is inhibited by $H_2O_2$, and Cu/Zn-SOD is inhibited by both compounds [23].

## Intracellular trehalose quantification assay

To validate the results of gene expression under osmotic stress, an experiment was delineated with 4 treatments and 4 replicates per treatment to quantify the levels of intracellular trehalose. Treatments consisted of exposing Lxx cells to 7% (w/v) PEG 6000 for 60 min and 72 h. Two controls were used representing cells not exposed to the agents and analyzed at the same time points. To this end, 8 mL (1% v/v) of a bacterial start culture ($O.D._{(600nm)}$ = 0.7, approximately 8 days) were added to 800 mL

**Table 1** Selected genes of *Leifsonia xyli* subsp. *xyli* for expression analysis in response to *in vitro* exposure to $H_2O_2$ and PEG6000 and their second BLAST best hits and corresponding *e* value and covering

| Metabolic process | Gene | Product | BLAST best hit | *e* value | Query cover (%) |
|---|---|---|---|---|---|
| Oxidative stress | *katA* | Catalase | *Leifsonia xyli* strain SE134 | 0.0 | 99 |
| | *ahpC* | Alkyl hydroperoxide reductase | *Leifsonia* sp. 21MF | 8.00E−129 | 98 |
| | *sodA* | Superoxide dismutase | *Leifsonia* sp. 21MF | 0.0 | 100 |
| | *bcp* | Peroxiredoxin | *Leifsonia* sp. 98AMF | 2.00E−115 | 99 |
| | *gpoA* | Glutathione peroxidase | *Leifsonia* sp. 21MF | 3.00E−128 | 85 |
| | *msrA* | Methionine sulfoxide reductase | *Leifsonia xyli* strain SE134 | 4.00E−162 | 97 |
| | *msrB* | Methionine sulfoxide reductase | *Sphingonomas* sp. MM-1 | 5.00E−26 | 64 |
| Osmotic stress | *otsA* | Trehalose-6-phosphate synthase | *Leifsonia* sp. 98AMF | 0.0 | 99 |
| | *otsB* | Trehalose-phosphatase | *Leifsonia* sp. 21MF | 1.00E−108 | 98 |
| | *treY* | Maltooligosyl trehalose synthase | *Leifsonia* sp. 21MF | 0.0 | 96 |
| | *treZ* | Maltooligosyl trehalose trehalohydrolase | *Leifsonia* xyli strain SE134 | 0.0 | 79 |
| | *treS* | Trehalose synthase | *Leifsonia* sp. 21MF | 0.0 | 99 |
| | *proW* | Permease protein, glycine betaine ABC transporter | *Leifsonia* xyli subsp. *cynodontis* | 0.0 | 95 |
| | *proZ* | Permease protein, glycine betaine ABC transporter | *Leifsonia* sp. 21MF | 6.00E−137 | 88 |
| | *proX* | Substrate-binding protein, glycine betaine ABC transporter | *Leifsonia* sp. 21MF | 0.0 | 96 |
| | *naggn* | N-acetylglutaminylglutamine amide | *Agromyces aureus* | 3.00E−104 | 93 |
| Normalizers | 16S *rRNA* | 16S ribosomal RNA | *Lysimonas* sp. LM-2018 | 0.0 | 100 |
| | *proC* | Pyrroline-5-carboxylate reductase | *Ketobacter alkanivorans* strain GI5 | 1.00E−09 | 7 |
| | *pyk* | Pyruvate kinase II | *Leifsonia* sp. 98AMF | 0.0 | 96 |
| | *qcrA* | Iron-sulfur component of ubiQ-cytB reductase | *Leifsonia xyli strain* SE134 | 0.0 | 99 |
| | *hrdB* | sigma-70 family RNA polymerase sigma factor | *Leifsonia xyli* strain SE134 | 0.0 | 78 |
| | *sigH* | RNA polymerase sigma-70 factor, EFC subfamily | *Leifsonia xyli* strain SE134 | 0.0 | 85 |
| | *fliA* | RNA polymerase sigma factor | *Curtobacterium* sp. BH2-1-1 | 1.00E−34 | 42 |

of fresh MSC culture medium and then divided into equal volumes of 50 mL in sixteen 125-mL Erlenmeyer flasks. Bacterial cultures were incubated for 7 days until they reached an O.D.$_{(600nm)}$ = 0.6 when 3.5 g (7% w/v) of PEG 6000 was added. Cells were collected by centrifugation at the defined time points in a benchtop microcentrifuge cooled at 4 °C and then standardized by weight. Cells were resuspended in 500 μL of 10 mM phosphate buffer pH 6.0. Cells were disrupted by sonication (40% amplitude, 0.5-s cycle, 10 cycles) and the supernatant was collected by centrifugation at 4 °C [25]. Trehalose was quantified using the Trehalose Assay K-TREH Kit (Megazyme). The concentration of trehalose is given in mg/L of the solution. Means were compared by the Tukey's test ($p$ = 0.05) using Assistat v 7.7 [26].

## Results

### *In silico* analysis of oxidative and osmotic stress-related genes

Keyword searches on the annotated sequence of the genome of Lxx CTCB07 unveiled several genes related to oxidative and osmotic stresses whose identities were confirmed by the BLAST analysis (Table 1, Supplementary Material). As expected, these genes shared the highest similarity with those of *L. x.* subsp. *cynodontis*, a pathogen of bermudagrass (*Cynodon dactylis*) and the second-best hits (Table 1) were with diverse *Leifsonia* spp. Of the oxidative stress response genes, three coding for haem peroxidases, being one catalase (*katA*) and two dye-decolorizing peroxidases (DyP-type peroxidases) and three coding for the non-haem peroxidases alkylhydroperoxidase (*ahpC*), bacterioferritin co-migratory protein (*bcp*), and glutathione peroxidase (*gpoA*) were identified. In addition, the genome of Lxx harbors two methionine sulfoxide reductase-encoding genes (*msrA* and *msrB*) and one superoxide dismutase (*sodA*) supposed to code for Mn-SOD based on predicted motifs (Supplementary Figure 1). Regarding the osmoprotective mechanisms, Lxx has genes of the three biosynthetic pathways of trehalose described in prokaryotes [27], the *otsAB*, *treYZ*, and *treS* genes. In addition, it has the *proW*, *Z*, and *X* genes of the osmoregulatory *proU* operon reported to encode three proteins that constitute a high-affinity glycine betaine transport system from external sources to the interior of the cell [28, 29]. Lxx also has a copy of *naggn* that encodes the dipeptide osmolyte N-acetylglutaminylglutamine amide (*naggn*) (Table 1).

## Quantification of lipid peroxidation of Lxx subjected to $H_2O_2$ and PEG 6000

Exposure both to PEG and $H_2O_2$ for 60 min increased cell lipid peroxidation compared with the unexposed control treatments (controls 0 and 60) (Fig. 1). However, PEG induced a greater level of stress compared with $H_2O_2$ as the levels of MDA in cells exposed to this compound (PEG6000 = 2.29 nmol MDA/mL) increased approximately 200 and 170% respectively to the C (control 0) and C60′ (control 60) control treatments, (C = 0.76 nmol MDA/mL and C60′ = 0.84 nmol MDA/mL) whereas exposure to $H_2O_2$ increased the levels by approximately 107 and 88% ($H_2O_2$ 60′ = 1.58 nmol MDA/mL). There was no difference between the control treatments, indicating that either one could be used in subsequent experiments. Therefore, the adopted control consisted of unexposed Lxx cultures analyzed immediately before the addition of the compounds to the medium (Control 0).

## Gene expression after exposure to $H_2O_2$ and PEG 6000

Gene expression analyses in response to $H_2O_2$ or PEG 6000 were performed for 16 of the 18 genes identified *in silico* (Fig. 2) after determining their optimal amplification conditions (Supplementary Table 1). Their minimum amplification efficiencies were 90%. The expression of the two DyP-type genes was not assessed because the role of this enzyme group is still unknown in the actinobacteria [12]. The *16S rRNA* (16S ribosomal RNA subunits) and *proC* (pyrroline-5-carboxylate reductase) genes were chosen as normalizers because they presented the lowest geometric average across the tested genes (Supplementary Figure 2).

Hydrogen peroxide induced the upregulation of *katA* at 30′ and 60′ of exposure and of *sodA*, *msrA*, and *msrB* at the three exposure time points compared to the control while no alteration in expression was detected for *ahpC*, *gpo*, and *bcp* (Fig. 2a). Contrastingly, all the osmoresponsive and general stress-responsive genes were downregulated in at least one of the three time points (Fig. 2a). The expression pattern after exposure to PEG contrasted with the one to $H_2O_2$ as the oxidative stress-related genes *katA*, *sodA*, *gpoA, msrA*, and *msrB* were downregulated in at least one time, while all the osmoresponsive genes were upregulated at all three time points (Fig. 2b).

## PAGE activities of CAT and SOD

A variation in the activity of CAT and SOD was detected in the presence of $H_2O_2$ compared with the control (Fig. 3a). For CAT, isoforms IV and VI were detected both in the control and 30′ after exposure to hydrogen peroxide. After 60′, a clear change in pattern by an intense increase in CAT activity with new isoenzymes being expressed was observed. For instance, in addition to isoform IV, isoform I was also detected together with a broad band at the end of the gel that may correspond either to isoform V or VI or both. At 120′, isoforms I and IV were inhibited and isoforms II and III were induced. It was also possible to clearly distinguish isoforms V and VI. For SOD, isoforms I, II, and IV were identified in the control but isoforms I and IV were inhibited and isoform III was induced at 30′. At 60′, all previous isoforms were inhibited and a new isoform (V) was induced and at 120′, this isoform was inhibited and isoforms II and III were induced again.

In contrast to the dynamic changes in the activities of these enzymes in response to hydrogen peroxide, the activity profiles of both enzymes did not change in the presence of PEG 6000 (Fig. 3b). Besides, the activity of SOD was not inhibited

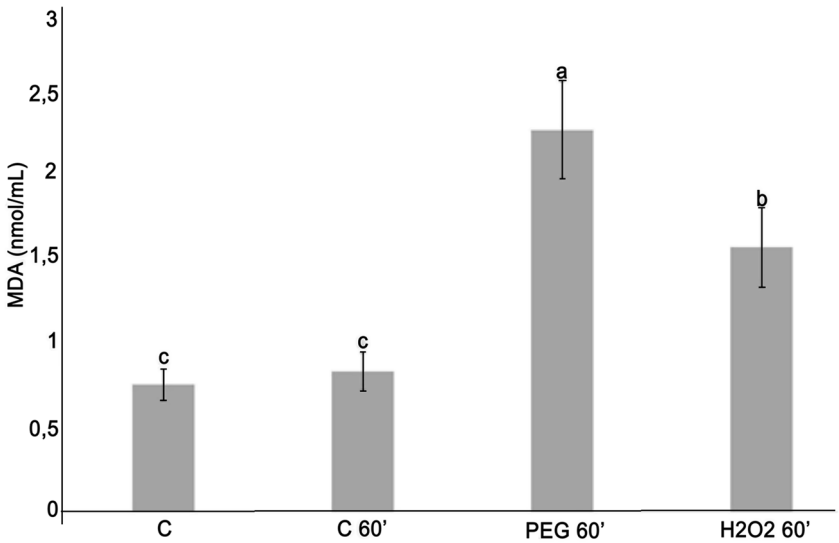


**Fig. 1** Concentration of malondialdehyde (MDA; nmol/g) in Lxx cells not exposed to 7% (w/v) PEG 6000 or 30 mM $H_2O_2$ at the initial time (C) and 60 min after the beginning of the experiment (C 60′) or exposed for 60 min to either compounds (PEG 60′; $H_2O_2$ 60′). Vertical lines represent the standard deviation of the means. Means with the same letter do not differ by Tukey's test ($p = 0.05$)

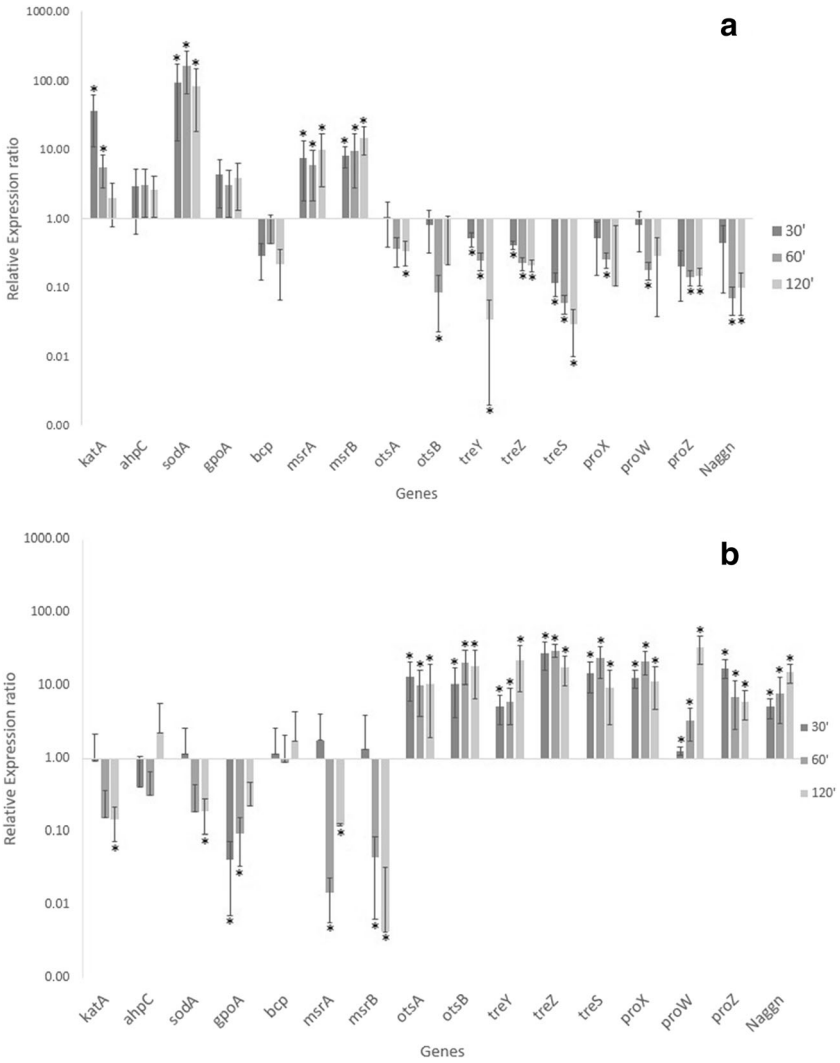


**Fig. 2** Relative expression ratio of Lxx genes after exposure to $H_2O_2$ (**a**) and PEG6000 (**b**). Single asterisk indicates significant differences ($p \leq 0.05$). *katA* catalase, *ahpC* alkyl hydroperoxide reductase, *sodA* superoxide dismutase, *bcp* peroxiredoxin, *gpoA* glutathione peroxidase, *msrA* methionine sulfoxide reductase, *msrB* methionine sulfoxide reductase, *otsA* trehalose-6-phosphate synthase, *otsB* trehalose-phosphatase, *treY* maltooligosyl trehalose synthase, *treZ* maltooligosyl trehalose trehalohydrolase, *treS* trehalose synthase, *proX* substrate-binding protein, glycine betaine ABC transporter, *proW* permease protein, glycine betaine ABC transporter, *proZ* permease protein, glycine betaine ABC transporter, *naggn* amidotransferase.

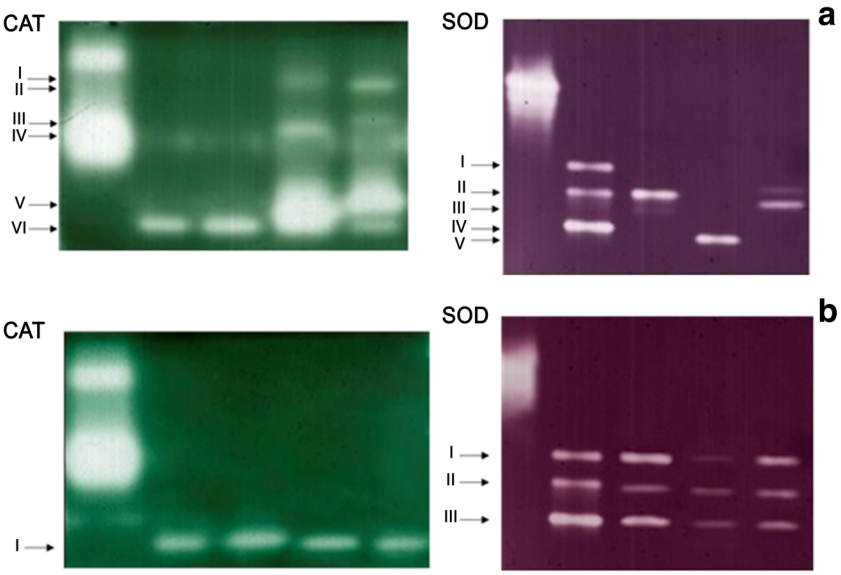


**Fig. 3** PAGE activities of catalase (CAT) and superoxide dismutase (SOD) of Lxx cells exposed to $H_2O_2$ (**a**) or PEG 6000 (**b**). P - bovine liver standards for each enzyme, C - control treatment just before exposure to the compounds and 30′, 60′, and 120′ min after

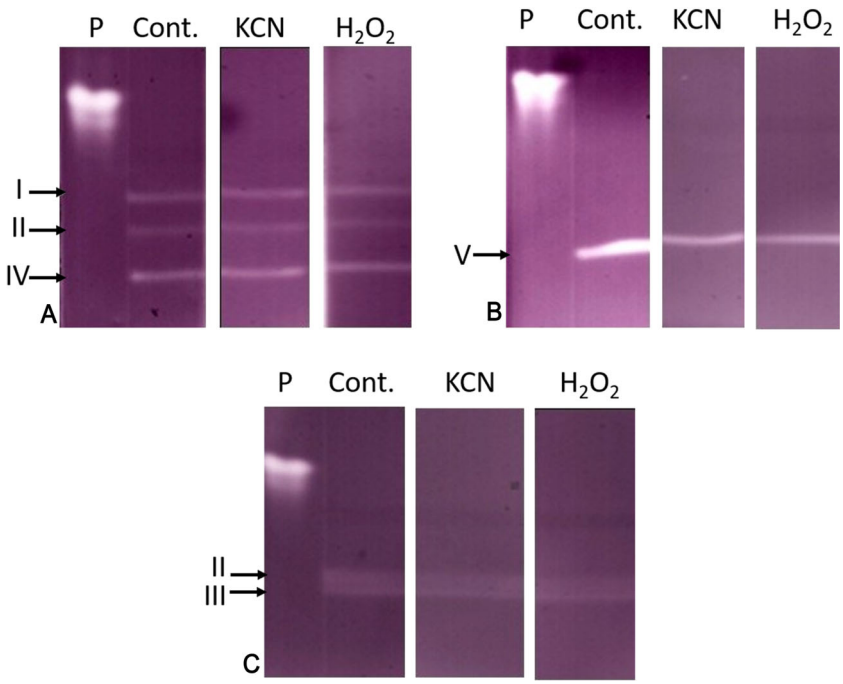


**Fig. 4** Inhibition tests of the activity of superoxide dismutase (SOD) of Lxx with 2 mM KCN and 5 mM $H_2O_2$ just before the exposure of Lxx cells to 30 mM of $H_2O_2$ (**a**) and 60 (**b**) and 120 min (**c**) after. P - bovine liver standards for each enzyme, Cont. - enzymes not treated with KCN or $H_2O_2$

after treatment with KCN or $H_2O_2$, indicating that Lxx produces Mn-SOD (Fig. 4).

### Quantification of intracellular trehalose production

The concentration of trehalose changed as a function of time as the concentration of this sugar was 70% higher at 72 h than at 60′ (0.0330 mg/L and 0.01975, respectively) in Lxx cells not exposed to PEG (Fig. 5). In cells exposed to this compound, the concentration did not significantly change after 60′ of exposure but reached levels 140% and 44% higher than in non-exposed cells at 60′ (0.0475 compared with 0.01975 mg/L) or 72 h (0.0475 compared with 0.033 mg/L), respectively.

## Discussion

The *in silico* analysis of the complete genome of *Leifsonia xyli* subsp. *xyli* CTCB07 unveiled genes related to common resistance mechanisms to oxidative and osmotic stresses and the study confirmed that they are expressed in all cases and most of them are responsive to these known stressing agents under the tested experimental conditions, except for *gpoA*, *ahpC*, and *bcp*.

As MDA is a byproduct of lipid peroxidation and an indicator of damage to the membrane that decreases its fluidity and affects the viability of the cell [1], the stressing effect of $H_2O_2$ and PEG6000 was first confirmed by measuring its levels after exposure to 30 mM of $H_2O_2$ and 7% (w/v) of

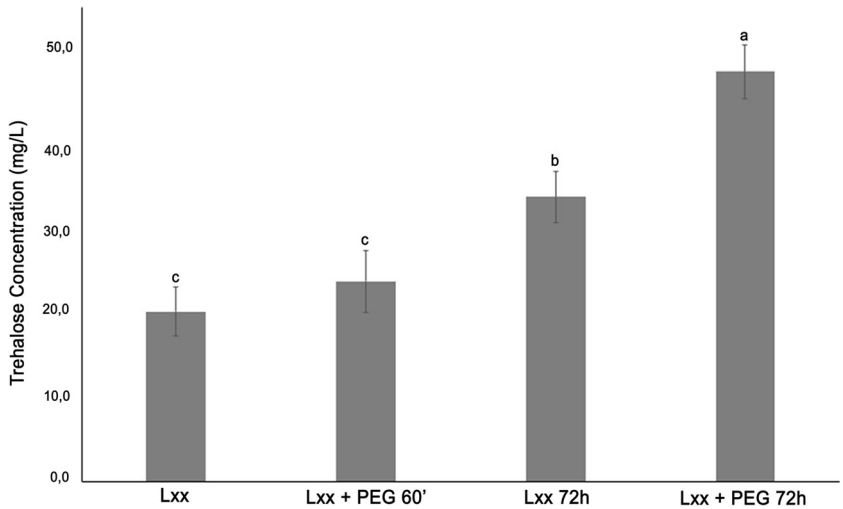


**Fig. 5** Concentration of trehalose (mg/L) in Lxx cells exposed to PEG for 60 min or 72 h (Lxx + PEG 60′ and Lxx + PEG 72 h, respectively). Lxx 60′ and Lxx 72-h treatments represent unexposed control cells at the same time points. Vertical lines represent the standard deviation of the means. Means with the same letter do not differ by Tukey's test ($p = 0.05$)

PEG 6000 for at least 60 min. On the other hand, in the absence of these compounds, no significant accumulation of MDA was detected in non-exposed control cells just before the beginning of the experiment or 60 min thereafter.

An interesting general expression pattern was observed between genes responsive to oxidative or osmotic stresses. In the first case, with the exception of *gpoA*, the oxidative stress-related genes that were upregulated in the presence of $H_2O_2$ were downregulated in the presence of PEG and the opposite was observed for genes of the second case, i.e., all osmotic stress-related genes upregulated in the presence of PEG were downregulated in the presence of $H_2O_2$, indicating the existence of a fine-tuned regulation of the stress-responsive systems that results in a balanced response to different stressing agents. In fact, despite sharing some components, each type of stress results in a specific response pattern because expression control is highly regulated to activate the defense systems only under stressful conditions [30, 31].

Exposure to $H_2O_2$ increased the expression of *sodA*, *katA*, *msrA*, and *msrB*. The first encode for superoxide dismutase (SOD), which converts superoxide radical ($O_2^-$) into $H_2O_2$ and oxygen in the presence of $H^+$. Major sources of ($O_2^-$) in the present case could result from the deprotonation of hydroperoxyl ($HO_2$) produced from the reaction between conjugated ferric iron of bovine hemin, an essential compound of the medium, and $H_2O_2$ (Fenton reaction) as well as from the peroxidation of lipids. The upregulation of *katA*, in turn, was required to decompose the hydrogen peroxide added to the medium and the one produced by the action of SOD into water and oxygen. *In vivo*, these enzymes would act not only against endogenous ROS but also against those produced by sugarcane. It has been reported [9] that ROS production, like in many other pathosystems, is an integral response of sugarcane to infection, as the activity of CAT, SOD, and of peroxidase (POX) was altered and the concentration of $O^{2-}$ increased in Lxx-inoculated plants. The activation of ROS defense mechanisms against Lxx was corroborated at the transcriptomic level [8]. The expression of the other three peroxidases (*ahpC*, *bcp*, and *gpoA*) was not altered by $H_2O_2$, indicating the preference of Lxx for *katA* as the major enzyme used to degrade hydrogen peroxide under the experimental conditions of the present study. In *E.coli*, for instance, AHP is the primary scavenger of endogenous rather than exogenous $H_2O_2$ [32] and BCP has a selectivity towards fatty acid peroxides instead of $H_2O_2$ [33]. As for the *gpoA* gene, it may be induced only when Lxx infects sugarcane and not *in vitro* because the bacterium lacks the genes necessary for the synthesis of glutathione (GSH) as many other actinobacteria [34], thus likely depending on the host as the major source of this antioxidant. Therefore, the basal levels of *gpoA* expression detected in this study could be achieved by scavenging GSH from the soybean tryptone of the medium. However, high levels of *gpoA* expression could not be sustained under this condition as the concentration of GSH is low in this peptone [35]. Alternatively, the expressions of *ahpC*, *bcp*, and *gpoA* may be induced at very early times after exposure and thus were not detected in this study.

Parallel to the alterations in the expression levels of *katA* and *sodA*, the activities of CAT and SOD also responded to $H_2O_2$. A clear increase in activity of the former enzyme was seen at 60 and 120 min of exposure in concert with the increased gene expression at 30 and 60 min. Isoforms IV and VI have constitutive expression and have been reported to control endogenous ROS, whereas the other isoforms detected at 60′ and 120′ protect against an excess of peroxide [36, 37]. The high relative expression of *sodA* at all three time points, however, was not followed by an overall increased activity of SOD in comparison to the control judging by the visual intensity of the bands. It is possible that some isoforms are more sensitive to inactivation by peroxyl radicals formed by the Fenton reaction [38]. However, the assays confirmed manganese as the metal cofactor of SOD as deduced from the *in silico* analysis.

Another detrimental effect of oxidative stress is the conversion of methionine into methionine sulfoxide (Met(O)) through the oxidation of the sulfur atom in the thioether side chain into sulfoxide. The *msr*A and *msr*B genes encode two methionine sulfoxide reductases, which reduces both free or proteic-Met(O) to Met [13]. The accumulation of Met(O) in prokaryotic cells can lead to enhanced ROS generation, reduced capacity to combat oxidative stress, protein degradation, and deactivation of several enzymes [13]. Having an efficient system to repair methionine residues seems to be crucial for Lxx as its growth *in vitro* is greatly improved by the addition of this amino acid probably due to the predicted non-functional mutations in genes of its synthetic pathway [10]. The upregulation of the methionine synthase gene of sugarcane in response to Lxx and the proposed sequestering of Met by the bacterium represents another mechanism by which Lxx could maintain the necessary supply of this amino acid [8].

The quantification of gene expression in the presence of PEG 6000 showed that all analyzed osmotic stress-related genes were upregulated in at least one of the three time points. Under osmotic stress, bacteria can accumulate osmolytes to rehydrate and maintain their viability. These compounds can be produced endogenously or sequestered from the environment by transporter proteins and then degraded or exported from the cell when no longer required. Trehalose is an ubiquitous non-reducing dissacharide and a neutral solute that stabilizes the conformation of proteins in their native states [27]. *Leifsonia xyli* subsp. *xyli* has three (OtsAB, TreYZ, and TreS) of the four pathways involved in the synthesis of trehalose described in prokaryotes [39] according to our *in silico* analyses and all were upregulated at the three time points in the presence of PEG. The gene responsible for the fourth pathway (TreT) which encodes trehalose glycosyltransferase and produces

trehalose from ADP-glucose and glucose has been described in hyperthermophilic bacteria [39] but is absent in Lxx. The OtsAB system involves the condensation of glucose-6-phosphate mediated by *ots*A (trehalose-6-phosphate synthase) into trehalose-6-phosphate which is dephosphorylated to yield trehalose by the product of *ots*B (trehalose-6-phosphate phosphatase). The second pathway uses *tre*Y (maltooligosyl trehalose synthase) to convert maltosyl residues into threhalosyl residues through transglycosylation followed by hydrolysis mediated by *tre*Z (maltooligosyl trehalose hydrolase) to liberate trehalose and the third converts maltose into trehalose by trehalose synthase encoded by *tre*S. It is noteworthy that Lxx has the complete biosynthetic pathway for the synthesis of maltose from glucose, and thus seems capable of synthesizing trehalose independently of external substrates provided by the host. However, since this bacterium is predicted to produce a maltose-/trehalose-binding protein coded by *malE*, it is also possible that under extreme conditions these sugars are recruited from the host to compound with the ones produced by the bacterium. The expression data together with the increased intracellular levels of trehalose in response to PEG also reported in the present study indicated that the accumulation of this osmolyte is an active response of Lxx.

In addition to trehalose, genes coding for the osmoregulated permease OpuC (ProU) and the dipeptide N-acetyl glutaminyl glutamine amide (Naggn) are also present in the genome of Lxx. In the first case, the system is represented by genes homologous to the *pro*X, *pro*W, and *pro*Z genes of Gram-negative bacteria [40] and transports osmoprotectants, such as glycine betaine. This compound can also be produced endogenously via the choline-betaine pathway, but since Lxx lacks the genes for the synthesis of choline, it probably relies on its host as a source of this osmolyte which highlights the importance of OpuC as an adaptative strategy. In fact, sugarcane genotypes have been reported to produce increased amounts of glycine betaine at differing levels under drought conditions [29]. The *naggn* gene encodes an amidotransferase able to convert N-acetyl glutaminyl glutamine into N-acetyl glutaminyl glutamine amide (Naggn). Naggn works along with other molecules previously described, such as trehalose and glycine betaine to oppose osmotic stress by accumulating in the cytosol [41].

Xylem-dwelling microorganisms like Lxx must cope with abiotic stresses present in this plant niche once the nutrient composition of the sap is poorer compared with the leaves and roots and the availability of oxygen is constrained by its structure [42]. The xylem sap is also prone to changes in osmolarity and in ionic composition as a function of water availability [43]. In addition, biotic stresses imposed by the plant host, notably the production of ROS, represent additional constraints to the infection and colonization processes. As a result of its genome evolution which resulted in a greater dependence on its host due to the accumulation of mutations [10], it is expected that the primary adaptative mechanisms here reported play a major role in the establishment of a successful parasitic relationship with sugarcane allowing Lxx to colonize the xylem of its host.

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## Compliance with ethical standards

**Conflict of interest** The author declares that there is no conflict of interest.

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