Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 2 (11): 838-844, 2007
ISSN 1816-4935

Sulphite Stress Induce Small Heat Shock Genes in Wine *Lactobacillus plantarum*

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Abstract: The expression of small heat shock (*shs*) genes in presence of sulphite, was monitored in MRS medium supplemented with 40, 60 and 80 mg L^{-1} of potassium metabisulphite ($K_2S_2O_5$). In all the experiments performed, the concentration of measured free sulphite was about 33% of the supplemented sulphite and no significant differences were observed between the pH values monitored though the experiment. Although high concentrations of sulphite (80 mg L^{-1}) reduce drastically the viability of *L. plantarum*, cells were able to tolerate sub-letal doses of sulphite (40 and 60 mg L^{-1}). RT-PCR revealed that the expression of *shs* genes analysed was different in presence of sulphite and was dependent from sulphite concentration. For all the *shs* genes an increase of cDNAs level was already observed at 40 mg L^{-1} of $K_2S_2O_5$. The expression of the *hsp* 18.5 and *hsp* 19.3 genes, compared with the cDNA control, remained unchanged or even decreased after the concentration of sulphite was increased at 60 or 80 mg L^{-1}, respectively. In contrast, *hsp* 18.55 expression increased at 60 mg L^{-1}. The transcripts of all the genes analysed decreased in MRS supplemented with 80 mg L^{-1} of $K_2S_2O_5$. These results suggest that a heat shock response like is probably confined to only some concentrations of sulphite in *Lactobacillus plantarum*.

Key words: Wine, sulphite, *Lactobacillus plantarum*, small heat shock genes

INTRODUCTION

In modern winemaking, sulphur dioxide, often abbreviated to sulphite or SO_2, is used extensively for its ability to suppress yeasts and bacterial response and its antioxidant properties. Most of the yeasts and bacteria found in wine are sensitive to sulphite, the only authorized and efficient agent for the stabilization of wine (Liu and Pilone, 1998; Lonvaud-Funel, 1999). Sulphite tolerance is considered a valued trait in selected wine strains used as starter such as *Oenococcus oeni* (Lonvaud-Funel, 1999; Liu, 2002). Of all the species of free sulphite, only undissociated sulphurous acid (H_2SO_3) possesses antimicrobial activity, presumably because it is able to cross the microbial cells membranes (Park and Bakalinsky, 2000). The undissociated forms of sulphite in wine are pH dependent. Lower the pH of a sulphite-containing wine, the more H_2SO_3 is present stronger will be its antimocrobial activity (Lonvaud-Funel, 1999).

Little genetic information exists on the effect of sulphite on wine Lactic Acid Bacteria (LAB). Malolactic fermentation (MLF) occurs in wine even in the presence of added SO_2. Consequently, LAB appear to be able to develop a tolerance to sulphite. How bacteria resist SO_2 is still unknown. Carrete *et al*. (2002) reported the inhibitory effect of sulphite on cell growth and malolactic fermentation (MLF) duration in *O. oeni*. This effects were related to an inhibition of ATPase activity (Carrete *et al*., 2002). Moreover, Guzzo *et al*. (1998) showed that acid-adapted cells of *O. oeni* survived better than non-adapted cells in the presence of a high sulphite concentration (30 mg L^{-1}).

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The presence of 60 mg L^{-1} of sulphite induced the expression, in *O. oeni*, of a small heat shock gene named *Lo18* and the authors suggested that stress protein synthesis could be involved in the induction of sulphite tolerance in *O. oeni* (Guzzo *et al*., 1997, 1998).

Lactobacillus plantarum is frequently isolated from red wine undergoing malolactic fermentation (MFL) and sterilised with sulphite (Spano *et al*., 2002; Beneduce *et al*., 2004) and it usually contributes to production of undesirable products such as histamine and precursors of ethyl carbammate (Lonvaud-Funel, 1999; Spano *et al*., 2004a, 2005, 2006a). Therefore, *L. plantarum* is of general concern because of its spoilage nature. We have recently observed a survival of *L. plantarum* in wine must supplemented with 80 mg L^{-1} of $K_2S_2O_5$ suggesting that also spoilage microorganisms such as *L. plantarum* have developed mechanisms able to escape or tolerate high doses of sulphite (Spano *et al*., 2007).

We have previously reported the characterization of three small heat shock genes (named *hsp* 18.5, *hsp* 18.55 and *hsp* 19.3) from wine *L. plantarum* and their regulation by abiotic stresses (Spano *et al*., 2004b, 2005). The aim of this study was to investigate the effect of different concentrations of sulphite on the expression of *L. plantarum* small heat shock genes.

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

All the experimental analysis reported in this study were performed during 2005-2006 season in the Department of Food Science at Foggia University, Foggia (Italy).

Lactobacillus plantarum strain Lp90, previously identified (Spano *et al*., 2004a), was grown without shaking at 28°C in an MRS broth (Oxoid, Basingstoke, UK) adjusted to pH 6.0.

Exponentially growing *L. plantarum* cells (optical density OD_{600} = 0.8) were diluted in 30 mL of fresh MRS broth supplemented with 40, 60 and 80 mg L^{-1} of $K_2S_2O_5$. The pH value was adjusted to pH 6.0 with KOH pellets. Total RNA was extracted after 2 h at 28°C. An uninoculated medium and an inoculated medium without $K_2S_2O_5$ were used as reference samples.

Free sulphite was measured with the methods of analysis CEE (1978) and the pH was monitored by a PH211 microprocessor pH-meter (Hanna Instrument).

In order to analyse sulphite tolerance, cells of *L. plantarum* (optical density OD_{600} = 0.8) were inoculated in 30 mL of fresh MRS broth supplemented with 40, 60 and 80 mg L^{-1} of $K_2S_2O_5$. Cells viability was then monitored for 10 h using both OD_{600} measurement and plate count analysis.

Total RNA Isolation and RT-PCR Analysis

Total RNA was extracted after 2 h of incubation in MRS media. RNA preparation was carried out using the NucleoSpin RNA II (Macherey-Nagel, Duren, Germany) which includes a DNAse (RNase-free) treatment, as recommended by the manufacturer. The quality of the RNA samples was checked on 1.2% (w/v) agarose gel and the concentration was determined by measuring the absorbance at 260 nm (GENEQUANT pro, Amersham). Synthesis of cDNA was carried out with the ProStar ™ ultra-HF RT-PCR System (Stratagene) using random primers available in the kit. About 100 ng of total RNA were used in a final volume of 50 µL for the RT-PCR experiments. The reaction mix was cycled through the following temperature profile: 45°C, 15 min (reverse transcriptase reaction); 95°C, 1 min; 95°C 30 sec (denaturation), 60°C, 1.2 min (annealing), 68°C, 30 sec: 40 cycles. The PCR reaction was terminated at 68°C for 5 min and thereafter cooled to 4°C. PCR fragments were visualized on 1.2% (w/v) agarose gel. Primers relative to the three small heat shock genes (named *hsp* 18.5, *hsp* 18.55 and *hsp* 19.3) were already reported (Spano *et al*., 2004b, 2005). PCR control included PCR with *23S* rRNA specific primers on water (negative control), RNA extracted from cultures in which the stresses were performed (negative controls) and genomic DNA instead of RNA (positive control).

The *L. plantarum 23S* rRNA gene was used as a constitutive expression control, since it is an housekeeping genes which is usually not induced by environmental stress. The primers corresponds to the internal portion of the *23S* rRNA gene isolated from the *L. plantarum* strain used in this study were already published (Spano *et al*., 2004a). In addition to the *23S* rRNA, the ftsH gene, encoding a protease belonging to the ATP binding cassette protein superfamily gene, which is apparently not induced by sulphite (Bourdineaud *et al*., 2003) was used as control marker and monitored through the sulphite stress. The *ftsH* gene was amplified with primers *ftshF* 5' GCAGCTACCTTCGAAGAATCCA 3' and 5 *ftshR* 5' GGGAAACTTGGTTCAGCAACA 3' deduced from the nucleotide sequences identified on *L. plantarum* WCFS1 (Kleerebezem *et al*., 2003). The experiment was repeated twice on cDNA made with mRNA extracted after independent isolation procedures.

Northern Blot Analysis and Densitometry Analysis

Twenty μg of total RNA were denatured and fractionated on a denaturing gel, prior to transferring to nylon membranes (Hybond N^+, Amersham). Hybridization was carried out using probes related to three small heat shock genes previously identified in wine *L. plantarum*. Moreover, in order to verify the amount of RNA loaded in each track and transferred onto the filters, the *23S* RNA and the *ftsH* genes were used as internal control. Filters were hybridised under standard conditions (Sambrook *et al*., 1989). Autoradiographic was performed by exposing membranes to Hyperfilm βmax (Amersham) using intensifying screens.

The probes were amplified from the pGEM-T easy vector and labelled with [α^{32}P] dCTP using a random primers DNA labelling kit (Invitrogen). Phosphor storage screens were scanned using the Bio-Rad Personal FX imager and densitometry was performed with Quantity One software (Bio-Rad).

RESULTS

Sulphite Tolerance in Wine *Lactobacillus plantarum*

Cells of *L. plantarum* grown in MRS medium at pH 6.0 were used as inoculum to study the survival of *L. plantarum* in the presence of 0, 40, 60 or 80 mg L^{-1} of sulphite (Fig. 1). Cells viability was then monitored for 10 h. As shown in Fig. 1, cell survival was markedly decreased after 10 h of incubation in the presence of sulphite. Total death was observed with 80 mg L^{-1} of sulphite. Within 6 h, almost all the cell died and no *cfu* was counted after this period of time. With 60 mg L^{-1} of sulphite, a decrease in cell population was observed after 3 h. However, viable cells were present even after 10 h. In the presence of 40 mg L^{-1} of sulphite, the number of viable counts after 10 h of incubation was only 2 log lower when compared to the control cells.

Taken together, these results indicates that, although high concentrations of sulphite (80 mg L^{-1}) reduce drastically the viability of *L. plantarum*, cells are still able to tolerate sub-letal doses of sulphite (40 and 60 mg L^{-1}).

Sulphite Stress Induce a Different Expression of the Small Heat Shock Genes Family

The expression of heat shock genes in presence of sulphite, was monitored in MRS media supplemented with 40, 60 and 80 mg L^{-1} of potassium metabisulphite ($K_2S_2O_5$). In all the experiments performed, the concentration of measured free sulphite was about 33% of the supplemented sulphite (Table 1).

RT-PCR revealed that the expression of the *shs* genes analysed was different in presence of sulphite and was dependent from sulphite concentration. For all the *shs* genes analysed, an increase of cDNAs level was already observed at 40 mg L^{-1} of $K_2S_2O_5$. The expression of the *hsp* 18.5 and *hsp*

Table 1: Changes of pH values and free sulphite in MRS supplemented with different concentration of $K_2S_2O_5$

Parameters	Time	Control	40 mg L^{-1} $K_2S_2O_5$	60 mg L^{-1} $K_2S_2O_5$	80 mg L^{-1} $K_2S_2O_5$
Free sulphite		Standard	12.80±0.24	18.50±0.20	25.20±0.30
	0 min	5.96±0.12	5.86±0.10	5.87±0.12	5.82±0.11
pH values	2 h	5.96±0.11	5.87±0.12	5.95±0.11	5.82±0.14

Prior total RNA extraction, the pH values of the medium was monitored over the course of the experiments. Each value is the mean value of three different measurement; This show the standard values

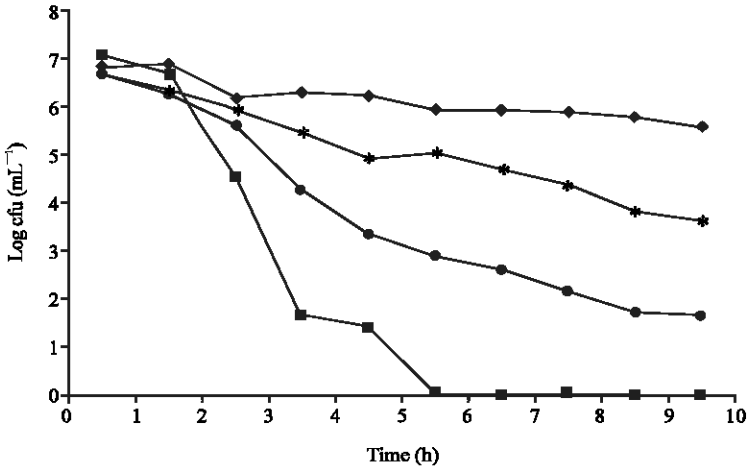

Fig. 1: Survival of wine *Lactobacillus plantarum* cells in the absence (C, ◆) or in the presence of various concentrations of sulphite: 40 mg L^{-1} (*); 60 mg L^{-1} (●); 80 mg L^{-1} (■). The data presented are mean values from two separate experiments

19.3 genes, compared with the cDNA control, remained unchanged or even decreased after the concentration of sulphite was increased at 60 or 80 mg L^{-1}, respectively (Fig. 2). However, when the expression of the *hsp* 18.55 gene was analysed, an increase of cDNA level was also observed at 60 mg L^{-1} (Fig. 2A). As reported for the *hsp* 18.5 and *hsp* 19.3 genes, a slight decrease in the *hsp* 18.55 cDNA level between control and stressed cells, was observed at 80 mg L^{-1} of $K_2S_2O_5$ (Fig. 2A). The expression patterns of the genes was compared to those obtained from two genes which fulfill separate and unrelated functions in *L. plantarum*. These include the ribosomal RNA (*23S*) an housekeeping gene which is usually not induced by environmental stress and the ftsH gene, encoding a protease belonging to the ATP binding cassette protein superfamily gene, which is apparently not induced by sulphite (Bourdineaud *et al*., 2003). As reported in Fig. 2, the expression of both the *23S* rRNA and the ftsH genes, was not significantly affected by sulphite. Controls were performed in order to verify that no DNA was extracted with RNA. PCR control included PCR with *23S* rRNA specific primers on water, RNA extracted from cultures with or without stress and genomic DNA instead of RNA (data not shown).

The RT-PCR analysis suggested that the genes analysed were differently induced by sulphite, with the strongest induction observed for the *hsp* 18.55 gene. Quantitative Northern blot analysis was therefore used to monitor the level of the *hsp* 18.5, *hsp* 18.55 and *hsp* 19.3 mRNAs. After 2 h at 40 mg L^{-1} of $K_2S_2O_5$ the *hsp* 18.55 transcript was six to seven times higher than in the control cells. The mRNA level was two to threefold higher at 60 mg L^{-1} of $K_2S_2O_5$ and was almost one time lower at 80 mg L^{-1} of $K_2S_2O_5$ (Fig. 2B). The pH value of the medium was monitored over the course of the experiments. No significant differences were observed between the pH values at the start and the end of the experiments (Table 1). Therefore, it is possible to argue that the result observed are related only to sulphite stress rather than pH stress.

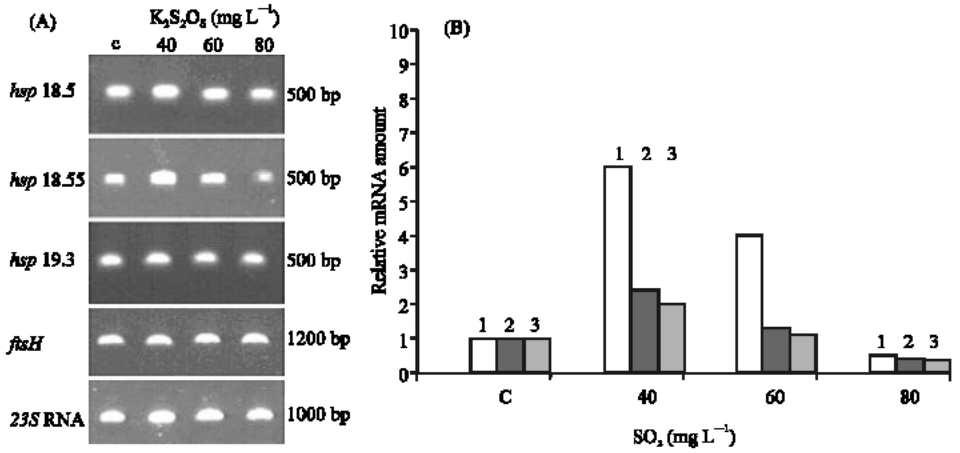

Fig. 2: RT-PCR and quantitative Northem blot analysis of *Lactobacillus plantarum hsp* 18.55, 18.5 and 19.3 genes expression. (A) RT-PCR obtained with cDNA made from mRNA of *L. plantarum* Lp90 strain extracted after 2 h of incubation in MRS media supplemented with 40, 60 and 80 mg L^{-1} of potassium metabisulphite ($K_2S_2O_5$). The size of the cDNA fragments amplified by RT-PCR is reported on the left. c: MRS without $K_2S_2O_5$ (control). (B) Relative mRNAs amount of *hsp* 18.55 (1), *hsp* 18.55 (2) and *hsp* 19.3 (3) in MRS media without $K_2S_2O_5$ (C) or supplemented with 40, 60 and 80 mg L^{-1} of $K_2S_2O_5$

All together, these results suggest that, although concentrations of sulphite of 40 mg L^{-1} are able to induce the expression of the *shs* gene family, concentration higher than 40 mg L^{-1} or between 40 and 60 mg L^{-1} induced the expression of only the *hsp* 18.55 gene in wine *L. plantarum*. Moreover, concentration of sulphite higher than 60 and/or between 60 and 80 mg L^{-1} seem to act as a sort of repressor on the expression of the *hsp* 18.5, 18.55 and 19.3 genes.

DISCUSSION

Beside the ability to undergoing biochemical process such as malolactic fermentation, the growth of wine LAB is strictly dependent on their capacity to tolerate multiple physical stresses. In fact, during vinification, several stress including an acidic pH, a high alcoholic content, non optimal growth temperatures, growth-inhibitory compounds such as fatty acids and tannins and sulphite treatment, may affect the survival of wine microorganism (Spano and Massa, 2006). Of the stress compounds analysed in wine so far, SO_2 and dodecanoic acid have recently been reported as the strongest stresses that may affect cell growth and malolactic fermentation (MLF) duration in *O. oeni* (Carrete *et al*., 2002).

In *O. oeni*, different stresses dramatically induce the expression of an 18 kDa small heat shock protein (named *Lo18*) that was found to be peripherally associated with the membrane. The expression of *Lo18*, was induced in *O. oeni*, by heat, ethanol, acid or sulphite shocks (Guzzo *et al*., 1997; Guzzo *et al*., 1998; Delmas *et al*., 2001) suggesting that the expression of this protein is a common response of *O. oeni* to hostile growth conditions (Zapparoli *et al*., 2004). Although *O. oeni* is the most important species among bacteria involved in MLF, studying the stress response in other wine LAB rather then *O. oeni*, may be important in order to understand either the ability of this microorganisms to compete with *O. oeni* in growth and survival or to tolerate multiple abiotic stresses.

In this study, we attempted to characterise the sulphite shock response of *L. plantarum*. Firstly, we observed that exposure of *L. plantarum* growing cells to 80 mg L^{-1} of sulphite, dramatically decreased the viability. However, viable *L. plantarum* cells were recovered when sub-lethal doses of sulphite were used suggesting that, in the tested conditions, wine *L. plantarum* is able to tolerate sulphite stress.

Using a molecular approach based on RT-PCR and Northern blot analysis, we have analysed in wine *L. plantarum*, the effect of different concentrations of sulphite on the expression of genes belonging to the small heat shock (*shs*) family previously characterized (Spano *et al*., 2004a, b). All the three *shs* genes analysed, were inducible at 40 mg L^{-1} of $K_2S_2O_5$. However, a different behaviour was observed when the concentration of sulphite was higher then 40 mg L^{-1}. The expression of the *hsp* 18.5 and *hsp* 19.3 genes, remained unchanged or even decreased after the concentration of sulphite was increased at 60 or 80 mg L^{-1} of $K_2S_2O_5$, respectively, while an increase of the *hsp* 18.55 gene was observed at 60 mg L^{-1} of $K_2S_2O_5$. The pH values of the media supplemented with sulphite was monitored through the experiments. However, no differences were observed between the pH values at the start and the end of the experiments suggesting that the induction observed was related to the stress imposed.

As reported for the *hsp* 18.5 and *hsp* 19.3 genes, a slight decrease in the *hsp* 18.55 cDNA level between control and stressed cells, was observed at 80 mg L^{-1} of $K_2S_2O_5$.

The different behaviour observed, may be ascribed to a different regulation of the small heat shock family isolated from wine *L. plantarum* (Spano *et al*., 2004b, 2005, 2006a). Quantitative Northern blot analysis showed that the *hsp* 18.55 transcript was six to seven times higher than the control cells and about three to four time higher than *hsp* 18.5 and *hsp* 19.3 cDNA in a medium supplemented with 40 mg L^{-1} of $K_2S_2O_5$. Moreover, the gene was also induced in medium supplemented with 60 mg L^{-1} of $K_2S_2O_5$. Concentration of sulphite higher than 60 mg L^{-1} of $K_2S_2O_5$ or between 60 and 80 mg L^{-1} of $K_2S_2O_5$ act as a sort of repressor of the *shs* genes in wine *L. plantarum*. Indeed, the transcripts of all the genes analysed decreased in a medium supplemented with 80 mg L^{-1} of $K_2S_2O_5$ suggesting that a heat shock response like is probably confined to only some concentrations of sulphite in wine *L. plantarum*. Furthermore, in *O. oeni* the *ftsH* gene is inducible by high temperature and osmotic stress (Bourdineaud *et al*., 2003) and the ftsH gene may be therefore considered a stress responsive gene. However, its expression was apparently unaffected by sulphite, suggesting that, among the stress response, sulphite have specific targets inside the bacterial cells and some heat shock proteins may be one of them.

Understanding the factors that affect sulphite tolerance or the basis of tolerance may be important in order to improve our knowledge on tolerance to abiotic stresses or to led towards a better management of sulphite use in wine making. We are extending our experiments on other stress responsive genes and several commercial strains of *O. oeni* which are supposed to be sulphite tolerance. Mutants or genes that are inducible by sulphite may help to elucidate the mechanisms which allow wine LAB to tolerate sulphite stress.

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