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Transcriptome analysis shows activation of the arginine deiminase pathway in *Lactococcus lactis* as a response to ethanol stress

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ABSTRACT

This paper describes the molecular response of *Lactococcus lactis* NZ9700 to ethanol. This strain is a well-known nisin producer and a lactic acid bacteria (LAB) model strain. Global transcriptome profiling using DNA microarrays demonstrated a bacterial adaptive response to the presence of 2% ethanol in the culture broth and differential expression of 67 genes. The highest up-regulation was detected for those genes involved in arginine degradation through the arginine deiminase (ADI) pathway (20–40 fold up-regulation). The metabolic responses to ethanol of wild type *L. lactis* strains were studied and compared to those of regulator-deletion mutants MGΔ*arg*R and MGΔ*ahr*C. The results showed that in the presence of 2% ethanol those strains with an active ADI pathway reached higher growth rates when arginine was available in the culture broth than in absence of arginine. In a chemically defined medium strains with an active ADI pathway consumed arginine and produced ornithine in the presence of 2% ethanol, hence corroborating that arginine catabolism is involved in the bacterial response to ethanol. This is the first study of the *L. lactis* response to ethanol stress to demonstrate the relevance of arginine catabolism for bacterial adaptation and survival in an ethanol containing medium.

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1. Introduction

Lactic acid bacteria (LAB) play an essential role in the process of fermentation of numerous foods and beverages (Bourdichon et al., 2012) giving rise to dairy products, meat and cereal-based foods (Kabak and Dobson, 2011), fermented vegetables (Hurtado et al., 2012; Settanni and Corsetti, 2008) and wine (Matthews et al., 2004; Mills et al., 2005). *Lactococcus lactis* has been associated with food production and preservation for centuries and it is by far the best studied of the food-related LAB, which is largely due to its major industrial importance as a starter in the manufacture of cheese. Its main activity during milk fermentation is the conversion of lactose to lactic acid, which results in the lowering of the pH in the product. Moreover, the capacity for lactate and bacteriocin production of *L. lactis* is beneficial for food preservation. During these food- and beverage related industrial processes, LAB can be exposed to a number of environmental stresses, among which low and high temperatures, oxidative stress, high osmotic pressure, acidity, nutrient starvation and the presence of ethanol are included. Growth performance and robustness to withstand environmental stresses are key properties for good starters. Bacterial mechanisms of stress resistance are based on bacterial adaptive responses and cross protection to those external factors. Advances in the genome, transcriptome and proteome research of *L. lactis* have turned this economically important LAB also into a widely used Gram-positive model organism (Pinto et al., 2011). *L. lactis* stress responses have been studied over the last years (Papadimitriou et al., 2016) and reports can be found on the response of *L. lactis* to osmotic stress (Sanders et al., 1998; Zhang et al., 2010), oxidative stress (Larsen et al., 2016; Miyoshi et al., 2003; Sheng et al., 2016), to both oxidative and acidic conditions (Cretenet et al., 2011), to acid stress (Budin-Verneuil et al., 2007; Carvalho et al., 2011; Carvalho et al., 2013; Hartke et al., 1996; Rallu et al., 1996; Sanders et al., 1995; Zhang et al., 2007), to heat- (Kim and Batt, 1993) and cold- (Panoff et al., 1994; Wouters et al., 2001) shocks, to starvation (Dressaire et al., 2011; Price et al., 2012) and to the presence of antibiotics (Dorrian et al., 2011). Cross-protective responses and interactive pathways have been demonstrated in a number of such responses of *L. lactis* to oxidative stress (Dijkstra et al., 2014; Duwat et al., 2000), osmotic, acid and thermal stress (Abdullah-al-Mahin et al.,

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2010; Van de Guchte et al., 2002; Zhang et al., 2014). Cross-protection induced by the expression of an adaptive response to one stress agent can be advantageous for bacterial tolerance to subsequent stress conditions; it increases the fitness of a bacterial culture to harsh conditions and will allow an optimal performance of a fermentative process carried out by this culture. Ethanol is a well-known antimicrobial agent, and tolerance to ethanol may be considered an indicator of bacterial robustness and might become a criterion for starter selection.

Arginine, a non-essential amino acid in *L. lactis*, can be synthesized de novo from glutamate in eight enzymatic steps, and is completely degraded into ornithine, ammonium and carbon dioxide via the arginine deiminase pathway (ADI pathway), which takes place in three enzymatic steps catalysed by the enzymes arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC). Arginine metabolism in *L. lactis* has been shown to be regulated by two transcriptional regulators named ArgR and AhrC (Larsen et al., 2004); both transcriptional regulators are required for repression of arginine biosynthesis in presence of the amino acid, and AhrC is an anti-repressor required to activate the ADI pathway of arginine degradation (Larsen et al., 2005).

This study aimed to identify the global adaptive response of *L. lactis* during growth in the presence of ethanol, which is a notorious stress factor for bacterial growth. Additionally, the bacteriocin nisin produced by some *L. lactis* strains had been previously reported to exert an inhibitory effect upon LAB strains isolated from wines and responsible for wine spoilage (Rojo-Bezares et al., 2007). The putative usage of a nisin-producer for wine preservation was an additional issue of interest for our study. Under these oenological conditions, ethanol exposure of wine LAB strains is a continuous and concentration-increasing exposure. We chose the model strain *L. lactis* subsp. *cremoris* NZ9700, which is a well-known nisin producer, its full genome had been sequenced and had been extensively studied (de Ruyter et al., 1996; Mu et al., 2015), nevertheless, no reports can be found on its response to ethanol. In this work we studied the molecular response of *L. lactis* NZ9700 to 2% ethanol exposure by whole-genome transcription profiling. To confirm and extend the obtained results, we then studied the arginine metabolism of the plasmid-free model strain *L. lactis* subsp. *cremoris* MG1363 and its single deletion mutants MGΔ*arg*R and MGΔ*ahr*C, whose ADI pathways of arginine degradation are either expressed or repressed.

2. Materials and methods

2.1. Bacterial strains and media

L. lactis strains used in this study are listed in Table 1. *L. lactis* was grown at 30 °C in M17 broth (Terzaghi and Sandine, 1975) with 0.5% glucose as the carbon source (GM17). A chemically defined medium (CDM) was prepared as described by Larsen et al. (2004); CDM buffer containing 15 free amino acids (CDM15) was prepared as previously described (Larsen et al., 2004). Arginine (Merck-VWR, Llinars del Vallès, Spain) stock solution was made in distilled water; pH was set to 7.0 with HCl. Growth and cell density were determined by measurement of the optical density at 600 nm (OD_{600}) of the culture using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech, Cambridge, UK).

2.2. Transcriptome analysis using L. lactis DNA microarrays

RNA was isolated from cells grown to mid-exponential (OD_{600} = 0.4) and stationary phase (OD_{600} = 1) in GM17 with 0% and 2% ethanol. Cells were harvested by centrifugation at 12,000 × *g* for 2 min at 4 °C. Supernatants were discarded and cell pellets were immediately frozen in liquid nitrogen and stored at − 80 °C. Pellets were resuspended in 400 µl of $T_{10}E_1$ buffer (10 mM Tris-HCl pH 8.0, 1 mM Na_2-EDTA), and 50 µl 10% SDS (w/v), 500 µl phenol/chloroform: isoamyl alcohol (24/24:1) (Sigma-Aldrich Chemie, Zwijndrecht, Netherlands), 500 mg glass beads (50–105 µm of diameter, Fischer Scientific BV, Den Bosch, the Netherlands), and 175 µl Macaloid suspension (Bentone MA, Elementis Specialities Inc., Hightstown, NJ) was added. The Macaloid suspension was made as follows: 2 g macaloid was boiled for 5 min in 100 ml $T_{10}E_1$, cooled to room temperature, sonicated by bursts until a gel was formed, centrifuged and resuspended in 50 ml $T_{10}E_1$. Cells were disrupted by shaking twice for 45 s in a Biospec Mini-bead Beater-8 (Biospec, OK, USA). The cell lysate was cleared by centrifugation and 500 µl supernatant was extracted with 500 µl chloroform:isoamyl alcohol (24:1). Total RNA was isolated from the water phase using the High Pure RNA Isolation Kit (Roche Applied Science, Mannheim, Germany) according the manufacturer's instructions. All reagents used for RNA work were treated with diethylpyrocarbonate (DEPC) (Sigma-Aldrich, St. Louis, MO). RNA quantity was determined spectrophotometrically and RNA quality was verified on an Agilent Bioanalyzer 2100 using RNA 6000 LabChips (Agilent Technologies Netherlands BV, Amstelveen, the Netherlands). 20 µg total RNA was used for the synthesis of aminoallyl-dUTP-labelled copy DNA (cDNA) using SuperScript III Reverse Transcriptase (Life Technologies, Carlsbad, California, US). Aminoallyl-dUTPs-containing cDNA was subsequently labelled using CyDye-NHS-esters Cy3 and Cy5 (Amersham Biosciences Europe GmbH). Labelled DNA was purified using NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, GmbH & Co. KG, Germany). Hybridisation (16 h at 45 °C) of Cy-labelled cDNA was performed in Ambion Slidehyb 1 hybridisation buffer (Ambion Europe Ltd., Huntington, UK) on full-genome *L. lactis* NZ9000 DNA Microarray slides (Kuipers et al., 2002) supplemented with probes for the nisin biosynthesis-cluster genes. Slides were scanned using a GenePix Autoloader 4200AL scanner (Molecular Devices Corporation, Sunnyvale, CA).

2.3. DNA microarray data analysis

Slide images were analyzed using ArrayPro 4.5 (Media Cybernetics Inc., Silver Spring, MD) and the data processed and normalized using MicroPrep software (van Hijum et al., 2003) and following standard routines provided by GENOME2D software available at http://genome2d.molgenrug.nl/index.php/analysis-pipeline. For each DNA microarray experiment, at least three independent biological replicates and two technical replicates (dye-swaps) were performed to discard possible differences due to variations in Cy3/Cy5 hybridisation. Expression ratios were calculated and a gene was considered differentially expressed when a p value of at least < 0.05 was obtained and the expression fold-change was at least > 1.8.

Table 1
Bacterial strains.

Strains	Descriptions	Characteristics	Source of reference
NZ9700	*Lactococcus lactis* subsp. *cremoris*	Nisin producer	Kuipers et al., 1993
MG1363	*Lactococcus lactis* subsp. *cremoris*	Plasmid free strain	Gasson, 1983
MGΔ*arg*R	Deletion mutant *arg*R of *L. lactis* subsp. *cremoris* MG1363	Active ADI pathway	Larsen et al., 2004
MGΔ*ahr*C	Deletion mutant *ahr*C of *L. lactis* subsp. *cremoris* MG1363	ADI pathway not expressed	Larsen et al., 2004