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Fluorescent differential display analysis of *Lactobacillus sakei* strains under stress conditions

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ABSTRACT

Lactobacillus (*Lb.*) *sakei* is widely used as starter in the production process of Italian fermented sausages and its growth and survival are affected by various factors such as temperature, pH and salt concentration. We studied the behaviour of *Lb. sakei* strains under various growth conditions relative to acid, osmotic and heat stress treatments by a novel fluorescent differential display (FDD) technique. This study obtained the development and the optimization of a technique that allows the identification of genome expression changes, associated with differential microbial behaviour under different stress conditions with a better stress response definition and a better discrimination of starter cultures. DNA sequence information from the FDD products provided an important tool to assess and observe the response to a variety of environmental stimuli and the adaptation to bacterial stress.

Our work provided an innovative FDD method, with a high level of reproducibility and quality for studying and probing the knowledge of the relation between differential genome expression and different stresses tolerance.

1. Introduction

Lactobacillus (*Lb.*) *sakei* is a ubiquitous lactic acid bacterium (LAB) and is commonly associated with the food environment and it is one of the most important LAB in meat technology. Although it is naturally present at low levels in raw meat, grows rapidly in freshly prepared sausages, and the changes resulting from its metabolic activity make a major contribution to the final characteristics of the product (Champomier-Verges et al., 2001). *Lb. sakei* is recognized as one of the most important components of starter cultures used for production of fermented meat products, most notably raw fermented sausages, in Western Europe (Leroy et al., 2006). The prevalence of *Lb. sakei* in a variety of habitats indicates its potential to adapt to and/or to compete in different ecosystems. Recently, the genome of the sausage isolate *Lb. sakei* 23 K was studied, analyzed (Marceau et al., 2004) and then its sequence was published (Chaillou et al., 2005) and further investigated (Hufner et al., 2007) providing fundamental information on the genetic endowment of this organism.

The genome analysis revealed potential survival strategies, as well as metabolic properties that enable *Lb. sakei* to effectively compete in the raw meat environment. The existence of such unique features can be viewed as evolutionary adaptation to the meat environment (Chaillou et al., 2005). In contrast, little is known about the regulation of gene expression of *Lb. sakei* in various environments. It has been argued that genes showing greater expression in a particular ecosystem ("niche-specific genes") are more likely to contribute to ecological fitness than genes expressed equally across a range of environments. If this is true, then only a combined knowledge of genome features and specific gene expression is required for understanding the adaptive mechanisms of *Lb. sakei* to the meat environment (Hufner et al., 2007). Comparative gene expression techniques, such as differential display (Liang and Pardee, 1992) and RNA arbitrarily primed polymerase chain reaction (RAP-PCR) (Welsh et al., 1992), have now become routine to examine changes in gene expression. Such methodologies provide useful approaches to identify differently expressed transcripts and to compare the difference of cDNA fingerprints when different treatments and time periods are involved, using only small quantities of RNA (Lockyer et al., 2004). Differential display PCR (DD-PCR) was originally developed for use in the study of eukaryotic gene expression, and this continues to be its most common application (Liang and Pardee, 1992; McClelland et al., 1995). RAP-PCR utilizes an arbitrary primer at a low annealing temperature for cDNA synthesis reactions, so it may be used for amplification of RNAs that are not polyadenylated, such as bacterial RNA (Welsh et al., 1992). This technology has been applied to only a few prokaryotic systems (Wong and McClelland, 1994; Shepard and Gilmore, 1999; Du and Kolenbrander, 2000; Chia et al., 2001; Le Breton et al., 2007; Lechiancole et al., 2006). Fluorescent differential display (FDD), that represents the next logical progression which use labeled primers or direct incorporation of labeled dNTPs, have been widely used and have replaced radioactive detection in many procedures (Reinhardt et al., 1999; Cho et al., 2001). FDD, optimized

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using fluorochrome labeled anchor primers and higher dNTP concentrations in PCR, was shown to be essentially identical in both sensitivity and reproducibility to that of conventional DD. Improvements such as elimination of radioactivity, digital data acquisition and increased assay speed were goals that were successfully reached by the establishment of the FDD platform, representing a marked improvement over conventional DD (Liang et al., 2007). FDD technique was used in some studies (Ripamonte et al., 2005; Lockyer et al., 2004; Xu et al., 2005; Shimizu et al., 2002) and this methodology allows the examination of changes in gene expression in response to different situations without the need of any prior knowledge of genomic information or selecting candidate genes that may be involved in the stress mechanisms. In this way, novel genes may be identified, as already occurred in previous studies (Dong-Kug et al., 1997; Keiji et al., 1997; Xu et al., 2005; Lockyer et al., 2004).

The aim of this study was to develop and optimize the FDD method for analyzing the different expression fingerprints of *Lb. sakei* strains under different stress conditions in order to verify the applicability of the selected potential starters and to obtain a better stress response definition in this species.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Five strains of *Lb. sakei* (DBPZ0062, DBPZ0098, DBPZ0338, DBPZ0329 and DBPZ0416 of the culture collection of the Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Università degli Studi della Basilicata, Potenza, Italy) used in this study were isolated from traditional fermented sausages of Basilicata region and identified by phenotypic and molecular analysis and technological characterization (Parente et al., 2001; Bonomo et al., 2008). In Parente et al. (2001) a total of 414 LAB strains were randomly isolated and characterized using a set of 28 phenotypic tests. 34 clusters were identified at the 80% similarity level using hierarchical cluster analysis and then 10% of the isolates of each phenotypic cluster was randomly chosen to carry out further analyses. In Bonomo et al. (2008) the 49 selected strains were subjected to a molecular identification at species and strain level. ARDRA-PCR revealed that *Lb. sakei* was the dominating species, underlining how *Lb. sakei* is the most adapted species to the fermented sausages environment. The data obtained by RAPD fingerprinting technique showed the strong selective effect of the stringent typical conditions of the sausages manufacturing on the indigenous microflora, with the presence of very different biotypes inside of the same species. Moreover, the strains were characterized on the basis of salt tolerance, acid production ability, growth at different temperatures, proteolytic, antimicrobial, and nitrate reductase activities. The *k*-means procedure classified them in eight clusters on the basis of their technological profile and allowed to select five strains able to grow and acidify at low temperatures, with a good proteolytic activity, antimicrobial capacity and a strong ability to hydrolyze sarcoplasmatic proteins.

All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0.1% w/v ascorbic acid (Riedel-de Haën, Sigma-Aldrich, Milan, Italy) and routinely cultivated in MRS broth at 30 °C for 16 h, before the evaluation of stress response.

2.2. Stress treatments

Late-exponential phase cells grown overnight in MRS broth, pH 6.2, were harvested by centrifugation (12,000 rpm, 5 min) and washed twice in sterile saline solution (0.85% w/v NaCl). The cells were re-suspended in 2 ml of different media to a final $OD_{600} = 1.0$ to achieve the following stress conditions: a) MRS broth, pH 2.5 and 3.0 (adjusted with HCl, acid stress); b) MRS broth with 9% w/v NaCl (osmotic stress); c) MRS broth, pH 6.2, at different temperatures (heat stress). Cell suspensions were incubated for 30 min at 30 °C for acid and osmotic stresses and at 50, 55 and 60 °C for heat stress. Bacterial cells incubated at 30 °C for 30 min in 2 ml of MRS broth, pH 6.2, were used as controls.

2.3. Measurement of growth and survival

After exposure of *Lb. sakei* cells to different stresses, cell survival were investigated by measuring colony forming units (CFU) on MRS agar plates. Cell counts were determined by serial decimal dilutions with sterile saline/peptone water (8.5 g/l NaCl and 0.1 g/l bacteriological peptone), plating on MRS agar and incubation at 30 °C in anaerobic conditions. Moreover, cell suspensions were incubated at different stress conditions and bacterial growth was followed by reading OD at 600 nm until a plateau was reached, this took about 48 h.

2.4. RNA extraction and reverse transcription

Lb. sakei cells were grown overnight in MRS broth at 30 °C and exposed to different stresses. RNA was extracted from 2 ml of *Lb. sakei* culture using RNA isolation kit supplied by Gentra System, Inc. (Minneapolis, MN, USA) according to the manufacturer's instructions. Purified RNAs were suspended in 200 µl of DEPC 0.1% diethylpyrocarbonate-treated water and stored at −20 °C. RNAs concentrations were calculated by measuring absorbance at 260 nm using the NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies Inc.). Prior to reverse transcription (RT), RNA (2 µg of total RNA) was treated with 2 U/µl of RNase-free DNase (Ambion, Applied Biosystems, Austin, TX, USA), as described by the manufacturer, to achieve complete DNA removal. Then, the cDNA was synthesized using the ProSTAR™ First-Strand RT-PCR kit (Stratagene, La Jolla, CA, USA) as recommended. RT reactions were performed in a final volume of 150 µl that contained master mix, random primers and 10 µl of RNA by subsequent incubation at 80 °C for 3 min, at 42 °C for 2 h and at 16 °C for 2 h. After dilution, 2 µl of cDNA were used for PCR analyses.

2.5. Fluorescent differential display (FDD)

The FDD technique was carried out by using a single fluorescently labeled universal primer, the random 6-carboxyfluorescein labeled 5′-anchored M13 primer (5′-6-carboxyfluorescein (FAM)-GAGGGTGGCGGTTCT-3′). The PCR mixture (25 µl) consisted of 2 µl of diluted cDNA, 2.5 µl of 1X PCR buffer (EuroClone, Pero, Milano, Italy), 3 mM of $MgCl_2$ (EuroClone), 0.4 mM of each dNTP (EuroClone), 0.6 µM of the primer (Invitrogen Ltd, Paisley,UK), 2.5 U of *Taq* polymerase (EuroClone). PCR amplification was carried out in a Genius Techne Progene thermal cycler (Cambridge, UK) using the following program: initial denaturation at 94 °C for 1 min; 40 cycles of 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min; followed by a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on 2% (w/v) agarose gels (EuroClone) in 1× TBE at 100 V for 4 h. Gels were stained in 1× TBE buffer containing 0.5 µg/ml ethidium bromide (Serva Electrophoresis GmbH, Heidelberg, Germany) for 30 min. A 1 kb DNA ladder (EuroClone) was used as molecular weight and normalization gel standard.

2.6. Statistical analysis

All measurements about cell growth and survival were performed in three independent assays, and the mean log values as well as standard deviation were calculated (Fig. 1).

Absorbance data were plotted versus time for each strain and for each tested condition. Absorbance slopes and absorbance values, reached at the plateau at stationary phase, were used respectively for