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Engineered strains of *Streptococcus macedonicus* towards an osmotic stress resistant phenotype retain their ability to produce the bacteriocin macedocin under hyperosmotic conditions

Rania Anastasiou[a], Gonzalez Van Driessche[b], Effrossyni Boutou[c], Maria Kazou[a], Voula Alexandraki[a], Constantinos E. Vorgias[c], Bart Devreese[b], Effie Tsakalidou[a], Konstantinos Papadimitriou[a,c,*]

[a] *Laboratory of Dairy Research, Department of Food Science and Human Nutrition, Agricultural University of Athens, Iera Odos 75, 118 55 Athens, Greece*
[b] *Laboratory for Protein Biochemistry and Biomolecular Engineering, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium*
[c] *Department of Biochemistry and Molecular Biology, Faculty of Biology, National and Kapodistrian University of Athens, Panepistimioupolis-Zographou, 157 84 Athens, Greece*

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ABSTRACT

Streptococcus macedonicus ACA-DC 198 produces the bacteriocin macedocin in milk only under low NaCl concentrations (<1.0% w/v). The thermosensitive plasmid pGh9:IS*S1* was employed to generate osmotic stress resistant (*osmr*) mutants of *S. macedonicus*. Three *osmr* mutants showing integration of the vector in unique chromosomal sites were identified and the disrupted loci were characterized. Interestingly, the mutants were able to grow and to produce macedocin at considerably higher concentrations of NaCl compared to the wild-type (up to 4.0% w/v). The production of macedocin under hyperosmotic conditions solely by the *osmr* mutants was validated by the well diffusion assay and by mass spectrometry analysis. RT-PCR experiments demonstrated that the macedocin biosynthetic regulon was transcribed at high salt concentrations only in the mutants. Mutant *osmr*3, the most robust mutant, was converted in its markerless derivative (*osmr*3f). Co-culture of *S. macedonicus* with spores of *Clostridium tyrobutyricum* in milk demonstrated that only the *osmr*3f mutant and not the wild-type inhibited the growth of the spores under hyperosmotic conditions (i.e., 2.5% w/v NaCl) due to the production of macedocin. Our study shows how genetic manipulation of a strain towards a stress resistant phenotype could improve bacteriocin production under conditions of the same stress.

1. Introduction

Lactic acid bacteria (LAB) include the key bacterial species used in food fermentations. One of their most important properties recognized over the past decades is the ability to produce bacteriocins (Beshkova and Frengova, 2012; Cotter et al., 2005). The benign nature of food related LAB makes their bacteriocins appealing antimicrobials to be used against food spoilage and foodborne pathogens. Nisin and pediocin are approved food additives, while the application of several other bacteriocins in food processing has also been reported in the bibliography (Cotter et al., 2005). However, the direct addition of bacteriocins in food matrices has sometimes proved to be problematic for a number of reasons (Khan et al., 2010). For example, bacteriocins may have solubility issues in the food environment, they may be sequestered by different food ingredients and they may be susceptible to proteolytic enzymes present in foods. In addition, microorganisms in the food ecosystem may be naturally resistant to bacteriocins. Furthermore, large scale purification of bacteriocins may be a daunting task (Carolissen-Mackay et al., 1997). In practice, the in situ production of bacteriocins by the producer strain directly into the food matrix is preferred (Beshkova and Frengova, 2012). Several LAB strains have been suggested that can be used as bioprotective starters or adjuncts. Nevertheless, this approach is not without weaknesses either. The multiple stresses applied during food processing and storage may negatively influence the production of bacteriocins due to their effect on the physiology of the producer strain.

Streptococcus macedonicus ACA-DC 198 produces the bacteriocin macedocin that inhibits the growth of important spoilage or pathogen microorganisms of cheese (e.g., *C. tyrobutyricum*, *Clostrid-*

* Corresponding author. Fax: +30 210 529 4672.
E-mail address: kpapadimitriou@aua.gr (K. Papadimitriou).

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ium perfringens, *Brochothrix* sp. and *Bacillus* sp.) and for this reason it was suggested that the strain could be used as a protective adjunct (Anastasiou et al., 2009; Anastasiou et al., 2007; Georgalaki et al., 2002; Van den Berghe et al., 2006). Macedocin is a lantibiotic of the lacticin 481 group and the gene cluster responsible for its biosynthesis (*mcd*) has been described (Papadelli et al., 2007). The *mcd* regulon consists of two putative operons, the *mcdAÁ* × *A1MTFEG* encoding the structural genes of macedocin along with the maturation, transport and immunity functions, as well as the *mcdRK* coding for the two component system (TCS) regulating the transcription of macedocin. It has been previously established that the optimum temperature for macedocin production is far below the optimum for growth (i.e., 20–25 °C vs. 42.3 °C) (Van den Berghe et al., 2006). Specific macedocin production is maximal at pH 6.0, slightly more acidic than pH 6.4 that is optimal for growth, while the peptide is not produced at NaCl concentrations of 2.0 or 4.0% (w/v) (Poirazi et al., 2007; Van den Berghe et al., 2006). In this study, we investigated the effect of hyperosmotic stress on the growth of *S. macedonicus* ACA-DC 198 and on the production of macedocin. We employed *S. macedonicus* strain ACA-DC 198 whose genome was recently sequenced (Papadimitriou et al., 2014; Papadimitriou et al., 2012). We performed a genetic screen using the thermosensitive vector pGh9:IS*S1* (Maguin et al., 1996) to generate osmotic resistant mutants (*osmr*) of *S. macedonicus* for the first time. The *osmr* mutants were characterized and their ability to synthesize macedocin in high NaCl concentrations was assessed. To the best of our knowledge this is the first report showing how genetically acquired resistance to a stress can favor bacteriocin production under conditions of the same stress.

2. Materials and methods

2.1. Bacterial strains and growth conditions

S. macedonicus ACA-DC 198 was grown at 30 or 42 °C in MRS medium (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 200 mM morpholinepropanesulfonic acid (MOPS, Sigma, St. Lewis, MO) and adjusted to pH 7.0 (MRS-MOPS), as previously described (Papadimitriou et al., 2007). For bacteriocin production, the strain was grown at 42 °C in skimmed milk (10% w/v, Oxoid Ltd.) with 0.3% (w/v) yeast extract (Biokar, Beauvais, FR). Skimmed milk supplemented with yeast extract was sterilized by autoclaving at 121 °C for 5 min according to the instructions of the manufacturer. *Lactococcus lactis* subsp. *lactis* LMG 6890^T was grown in M17 broth (Biokar) at 30 °C for 18 h, while *C. tyrobutyricum* LMG 1285^T was grown in Reinforced Clostridial Medium broth (RCM, Biokar) at 37 °C for 48 h (inoculum of 1%, v/v) under anaerobic conditions (GasPak^{TM} EZ Anaerobe Container System, BD Diagnostics, MD, USA). *Escherichia coli* EC101 was the host for plasmid rescue experiments and was grown in Luria–Bertani (LB) medium in the presence of erythromycin (160 μg/ml). *E. coli EC101* and the *EC101* strain containing pGh9:IS*S1* were kindly provided by Prof. Ezio Ricca (Department of Biology, Federico II University, Naples, Italy).

Media for hyperosmotic treatment were prepared as follows: In the case of the synthetic medium, NaCl was directly dissolved in MRS-MOPS so as to reach concentrations ranging from 0.5–4.0% (w/v) prior to sterilization. In the case of skimmed milk, the same concentrations were achieved by aseptically adding the appropriate volume from a sterile 30% (w/v) NaCl solution in pre-sterilized milk media. The acidification of milk during the growth of *S. macedonicus* ACA-DC 198 in milk was recorded using a Hanna HI 98,249 pH meter (Woonsocket, RI, USA).

2.2. Transformation of Streptococcus macedonicus cells with pGh9:IS*S*1

To the best of our knowledge, electrotransformation of *S. macedonicus* has not been attempted before. The strain proved quite resistant to electroporation with pGh9:IS*S1*. According to recent *in silico* genomic evidence *S. macedonicus* ACA-DC 198 seems to be able to degrade foreign DNA as it is a member of the *Streptococcus bovis*/*Streptococcus equinus* complex that carries 7 restriction modification systems and a clustered regularly interspaced short palindromic repeats (CRISPR) system (Papadimitriou et al., 2014). We have attempted to clone additional plasmids in *S. macedonicus* e.g., the pORI198 that carries the origin of replication of a native *S. macedonicus* plasmid with no success (Papadimitriou et al., 2015). A procedure yielding some tens of transformants per μg of the pGh9:IS*S1* plasmid was developed relying partly on the Holo and Nes protocol (Holo and Nes, 1989) without growing the strain in osmotically stabilized with glycine medium. In detail, early logarithmic phase cells (OD_{620} ~0.3–0.4) grown in MRS-MOPS (37 °C) were harvested by centrifugation at 8000 rpm for 5 min and washed twice with ice-cold buffer containing 0.5 M sucrose and 10% (v/v) glycerol. Cells were re-suspended in the same buffer at a 20-fold concentration and kept on ice for no more than 30 min. Forty-microliter aliquots of competent cells were mixed with 1–5 μl of DNA dissolved in 5 mM Tris/HCl pH 8.5. Electroporation was performed using the MicroPulser^{TM} electroporator (Bio-Rad Laboratories, Hercules, CA, USA) at 2.5 kV, while resistance was set at 200 ohms and capacitance at 25 μF in cuvettes of 0.2 cm electrode gap width (O'Sullivan and Fitzgerald, 1999). Immediately after electroporation, the suspension was mixed with 960 μl of MRS-MOPS containing 0.5 M sucrose, 20 mM $MgCl_2$ and 2 mM $CaCl_2$. The cells were incubated at 30 °C for 1 h. Serial dilutions were plated on MRS-MOPS agar (1.5% w/v) containing 1 μg/ml erythromycin and colonies were selected after 48 h at 30 °C. Even with this protocol our transformation efficiency was very low and the success of the procedure erratic. Inability to efficiently transform "wild" LAB strains is not an uncommon event.

2.3. Isolation of Streptococcus macedonicus osmr mutants generated using the pGh9:IS*S*1 plasmid and subsequent characterization

pGh9:IS*S1* is a thermosensitive replicon that at low temperatures (permissive) can replicate in the cytosol of LAB like *L. lactis*, while shifting the conditions of the culture to higher temperatures (restrictive) initiates the integration of the plasmid in the chromosome of the host (Maguin et al., 1996). In general, the presence of the IS*S1* element in the vector supports its randomized integration in the genome. Both permissive (30 °C) and restrictive (42 °C) temperatures of the transposition assay in *S. macedonicus* were chosen according to the relevant literature for *L. lactis* and/or *Streptococcus thermophilus* (Baccigalupi et al., 2000; Maguin et al., 1996; Thibessard et al., 2002). The final restrictive temperature for the selection of the *osmr* mutants was determined in control experiments with the wild-type plated on MRS-MOPS plates in which the NaCl content was kept constant at 2.0% (w/v) and the incubation temperature was increased until cell death occurred at a rate of ~10^{-8}–10^{-9} within 72 h. This temperature was determined to be 46 °C. It should be mentioned that 46 °C was not lethal for the *S. macedonicus* wild-type in the absence of NaCl. The isolation of osmotic tolerant *S. macedonicus* mutants (*osmr*) relied basically on similar protocols described previously (Baccigalupi et al., 2000; Maguin et al., 1996; Thibessard et al., 2002), adapted for the particular strain and the particular stress. In detail, *S. macedonicus* transformed with pGh9:IS*S1* was grown overnight in MRS-MOPS with erythromycin (1 μg/ml). Fifty microliters were used to inoc-