Improvement of Phenotype Microarray protocol for chemical sensitivity analysis of *Streptococcus thermophilus*

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Abstract

Phenotype MicroArray (PM) permits the characterization of bacteria in nearly 2000 culture conditions. PM standard procedure for chemical sensitivity analysis of Gram-positive bacteria failed in the analysis of *Streptococcus thermophilus*, therefore we developed an efficient and reproducible protocol to obtain a chemical sensitive profile by PM of *S. thermophilus*.

Keywords: Phenotype MicroArray, *Streptococcus thermophilus*

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In the *"omics"* era one of the technological goal is to obtain the whole phonotypical profile of biotechnological bacterial strains. Phenotype MicroArray (PM) is a high-throughput technology that provides a set of nearly 2000 culture conditions (about 200 carbon sources, 400 nitrogen sources, 100 phosphorous and sulphur sources, 100 nutrient supplements, and a range of pH, ion and osmotic gradients) in which one can test the metabolic activity of a microorganism. PM uses 96-wells plates and each well of the plates is designed to test a different phenotype. The plates are inoculated with a standardized cell suspension and incubated, typically for 24-48 hours. PM is based on the tetrazolium violet reduction as a reporter of active metabolism (Bochner et al., 2001). The reduction of the dye causes the formation of a purple colour that is recorded by a CCD camera every 15 min and provides quantitative and kinetic information about the response of the cells in the PM plates (Bochner et al., 2001). The data obtained are stored in computer files and can be analyzed by statistical software or used to compare the PM kinetics of different strains. PM technology has been extensively used in global phenotypic characterization of bacteria and fungi (Viti et al., 2008; Bochner et al., 2008; Bochner, 2009). Unfortunately the PM analysis, under the standard conditions defined by Biolog Inc., is not applicable to determine the chemical sensitivity phenotypical profile of *Streptococcus thermophilus* for the incapability of the redox potential indicator to become coloured during the metabolic activity. *S. thermophilus,* albeit phylogenetically close to pathogenic streptococci, has a long history of safe use, and it is one of the most important lactic acid bacteria in the dairy industry, well known as a starter culture component in yoghurt fermentation and cheese-making. Bacterial strains can undergo phenotypic variation due to the different environmental pressures (Fortina et al., 1998) and the main risk related to the phenotypic variation of dairy strains consists in the acquisition of antibiotics and/or biocides resistance determinants that might be transfer to other food-borne bacteria or to pathogenic bacteria in the gastrointestinal tract (Mathur and Singh, 2005). Thus, efforts must be done in order to assess the chemical sensitivity pattern of strain isolated from dairy industry. In response to these issues here we developed a standard protocol in order to assay contemporaneously the activity of *S. thermophilus* under 960 (panels PM11-PM20, detailed information about PM panels is available at www.biolog.com) different chemical stress conditions (240 toxic chemical compounds at 4 different concentrations, including antibiotics, biocides, heavy metals, antimetabolites, ect.). Thus, experiments were attempted in order to define a suitable protocol (carbon source and concentration, dye and phosphate buffer concentration) for colour development of the redox potential indicator in response to the metabolic activity of *S. thermophilus* strains. Four strains of *S. thermophilus* named MIM21, MIM119, MIM578 and MIM581, have been isolated from yogurt and the type strain *S. thermophilus* DSM20617 isolated from pasteurized milk, were grown on M17 agar plates (2% v/w

lactose) at 37°C in anaerobiosis for 24 hours. Bacterial cells were picked from the agar surface with a sterile cotton swab and suspended in IF-0 (Biolog Inc.) until the cell density reached 80% transmittance on a Biolog turbidimeter. The bacterial suspension was diluted 13.64 times in a medium consisting of modified IF-10 GN/GP (Biolog Inc.) with addition of 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.005% (v/v) tween 85, 0.005% (w/v) yeast extract, 25 μM Hypoxantine-HCl, 5 μM β-NAD and 0.25 μM riboflavine, as suggested by Biolog Inc.. Therefore sucrose or lactose [that are preferred carbon and energy sources for *S. thermophilus* for instance in respect to glucose (van den Bogaard et al., 2000)] at different concentrations (0.025%, 0.05%, 0.1%, 0.2% and 0.3%, w/v of sucrose; 0.1%, 0.2% and 0.3%, w/v of lactose), and Na-phosphate buffer pH 6.5 at concentrations ranging from 0 to 30 mM (0, 5, 10, 20 and 30 mM) were tested in order to determine optimal condition for growth/activity of the strains. Two sets of microplates with media above described were setup, one was added with 1x dye G (Biolog Inc.) (a redox potential indicator that turns from uncoloured to violet when it is reduced by the bacterial metabolic activity), while another set was added 0.05% (w/v) bromocresolpurple (BCP) (a pH indicator that turns from dark blue to light yellow in the pH range 7.0-3.5). The latter was used in order to evaluate if the acidification of the medium due to bacterial metabolism can interfere with dye G indicator.

96 well microplates (each condition tested was triplicate) inoculated with *S. thermophilus* strains were incubated at 37°C in the Omnilog instrument. The colour images of each microplate were acquired by the CCD camera and subsequently converted to grey-scale equivalent by the Omnilog software and a kinetic curve describing the temporal variation of grey intensity in each well was produced within 24 hours of incubation. In order to evaluate the response of the dyes used in culture of *S. thermophilus* strains the parameter ΔH (Fig. 1), defined as the difference between maximum and minimum height of the kinetic curve observed through 24 hours ($\Delta H=H_{max}-H_{min}$), was used both for cultures added with BCP and for the cultures added with dye G.

The results obtained for the type strain *S. thermophilus* DSM20617 using different carbon sources, dye and buffer concentrations are reported in Fig. 2. The reduction of the dye G depended both on the pH of the medium and on the type and concentration of the carbon source. In media without Na-phosphate buffer the strong decrease of pH induced by the activity of the bacteria prevented the reduction of the dye G that failed in its function of redox potential indicator. The best condition for the redox potential indicator activity was obtained when the concentration of Na-phosphate buffer was enough to prevent the acidification of the medium. The more effective reduction of the dye G was obtained in medium added with 30 mM Na-phosphate buffer and the highest concentration of carbon sources (0.3%) that sustained a high bacterial metabolic activity.

Therefore dye G in presence of 30 mM Na-phosphate buffer is a valid indicator for PM analysis of *S. thermophilus.*

From the above reported observations we defined a suitable protocol for the PM-chemical sensitivity panels (PM11-PM20), which makes use of the following medium: IF-10 GN/GP (Biolog Inc.) added with 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.005% tween 85, 0.005% yeast extract, 25 μM Hypoxantine-HCl, 5 μM β-NAD and 0.25 μM riboflavine, 30 mM Na-phosphate buffer, 0.3% carbon/energy source and 1x dye G (Biolog Inc.).

The protocol was tested, in duplicate, to obtain a chemical sensitivity profiles (panels PM11-PM20) of five strains of *S. thermophilus* (MIM21, MIM119, MIM578, MIM581 and DSM20617). Although lactose or sucrose were suitable for PM analysis (Fig. 2), lactose was chosen as carbon source because it is the main carbon source for *S. thermophilus* in milk that is the natural environment for this bacterium. For all stains, were obtained reproducible and defined profiles. The comparison between kinetic curves obtained in response to 960 different conditions of strains MIM21 and MIM119 is reported in Fig. 3, as an example.

An approach widely used to analyze phenotypic profiles of microorganisms is principal component analysis (PCA). PCA is an exploratory method of data analysis which, through the calculation of linear combinations of original variables, allows the number of dimensions in the data set to be considerably reduced while maintaining most of the original information of the data set, expressed as percent variance, allowing bettering visualizing relationships between strains diversity (Gatti et al., 1999). PCA was applied (BioNumerics Applied Maths) to the PM data using area of the kinetic curves as parameter. Division by the variances over the strains was used in order to normalize the intensity of the characters for all the strains. The first principal component (which accounted for 49.7% of phenotypic variation) separated the reference strain DSM20617 from the industrial strains (MIM21, MIM119, MIM578, MIM581) (Fig. 4). The second component (which accounted for 26.7% of phenotypic variation) provided a fairly good separation of strains MIM21 and MIM578 from strains MIM119 and MIM581 (Fig. 4). This result shows that our strains isolated from Italian diary products are phenotypically different from type strain isolated from pasteurized milk. Moreover, the fact that strains MIM578, MIM 21, MIM119 and MIM581 were grouped in two different homogenous groups on the basis of PCA analysis (FIg. 4) confirmed the high phenotypic and genotypic variability previously observed in this species (Mora et al., 2002).

Concluding the protocol here reported showed that condition fluid defined can be satisfactory used to obtain a fast and wide characterization of the chemical sensitivity of reference and environmental strains of *S. thermophilus. Moreover* PM approach should be use as a tool to point

out chemical sensitivity variation of dairy strains useful to highlight dangerous modifications in the genome of microorganisms, such as antibiotic or biocide resistance, used in industrial processes.

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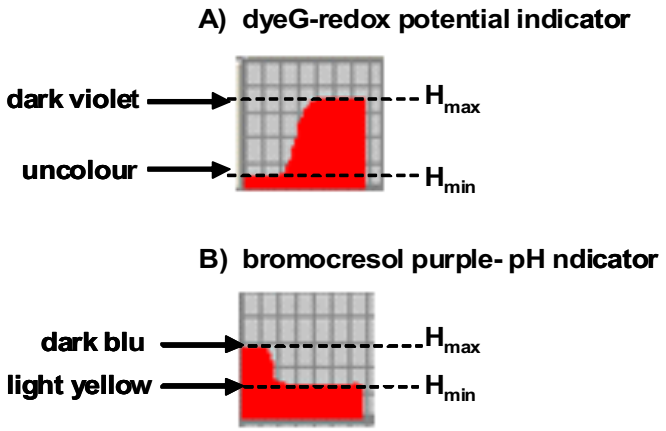
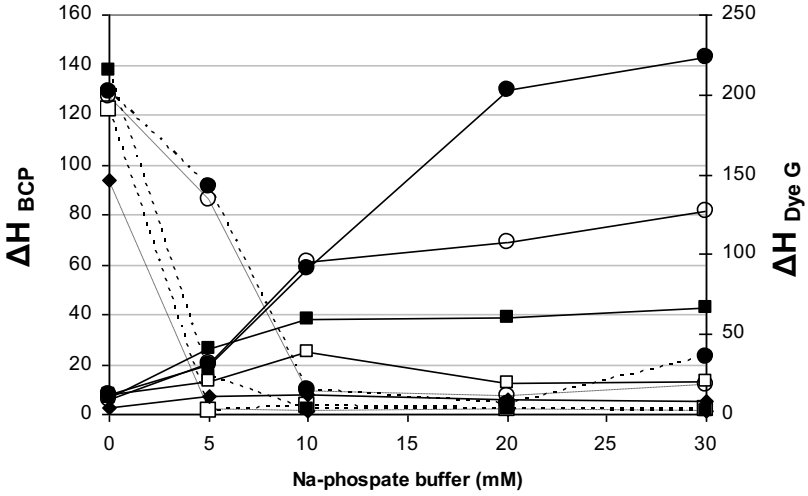


Fig. 1. Kinetic curves obtained from *S. thermophilus* DSM20617 in presence of dye G as redox potential indicator (A) or BCP as pH indicator (B). The PM software converts the colour image of the plate in the equivalent grey-scale image. In a metabolic active culture the dye G turns from uncoloured to violet and subsequently the kinetic curve increases with time (A), on the contrary the fermentative activity of the culture decreases the pH of the medium inducing the colour change of the pH indicator form dark blue to light yellow (B), consequently the kinetic curves decrease with time. The difference between the maximum and minimum height detected (ΔH) are a measure of the reduction of the dye G or of the pH decrease of the medium.

A)



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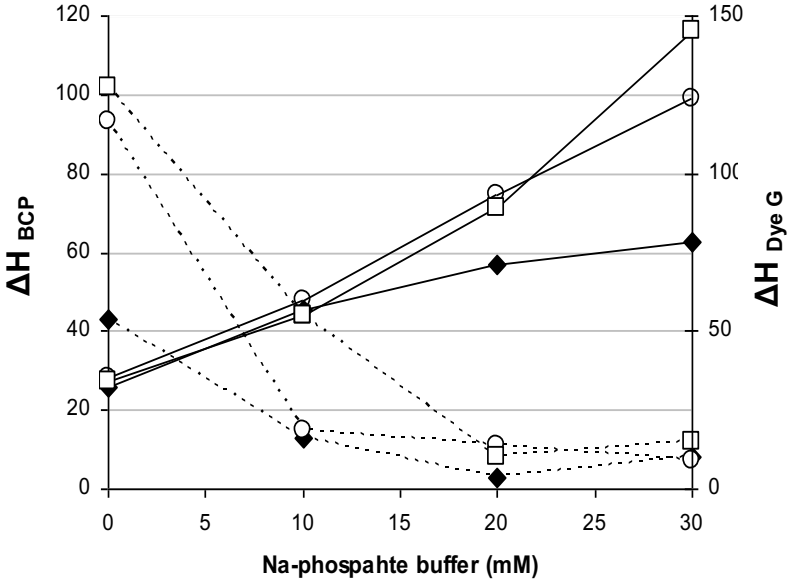
B)



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3 **Fig. 2.** Responses of the BCP (broken line) and dye G (continuous line) in cultures of type strain
4 DSM20617, in dependence of the sucrose (A) or lactose (B), in presence of different concentrations
5 of Na-phosphate buffer after 24 hour of incubation at 37°C. In the *y* axis the ΔH calculated for BCP
6 and dye G are reported. In A) ♦ = 0.025% sucrose, □ = 0.05% sucrose, ■ = 0.1% sucrose, ○ =
7 0.2% sucrose, ● = 0.3% sucrose. In B) ♦ = 0.1% lactose, ○ = 0.2% lactose, □ = 0.3% lactose.
8 Results are expressed as means of three independent determinations. Standard deviation is less than
9 1% of the given values.

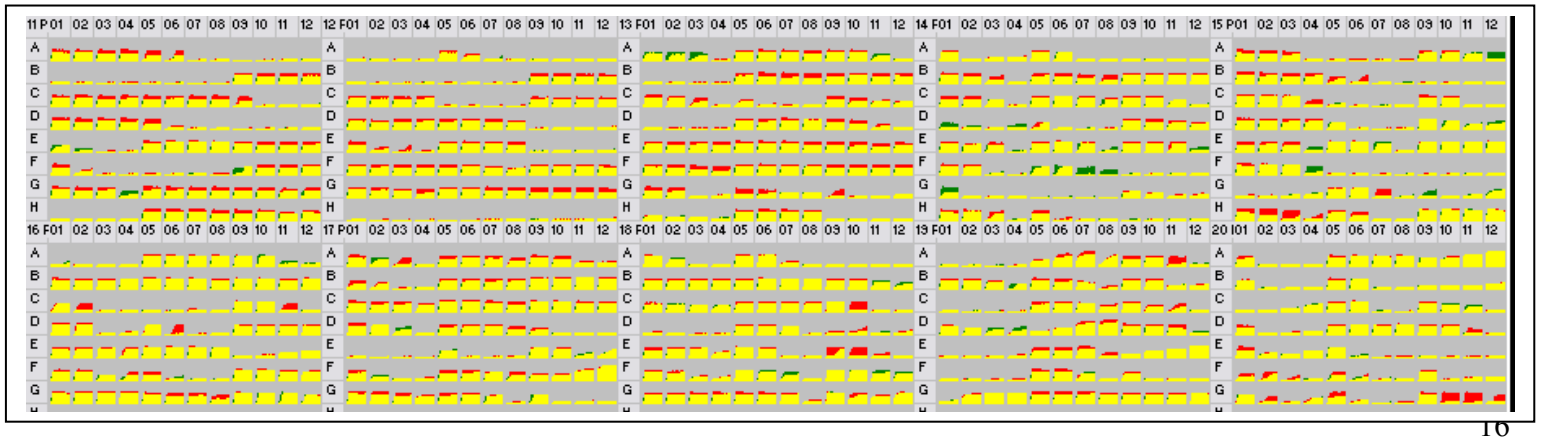

Fig. 3. PM chemical sensitivity profiles of MIM21 (green) and MIM119 (red) strains, obtained in 920 different conditions, are reported as example. Data, resulting after 48 h of incubation, are displayed in the form of kinetic graphs. Each square represents a single test. When the two strains reveal an equivalent metabolic activity in a well, the kinetic graphs overlap and are yellow. Green kinetic graphs indicate a stronger response by strain MIM21, red kinetic graphs a stronger response by strain MIM119.

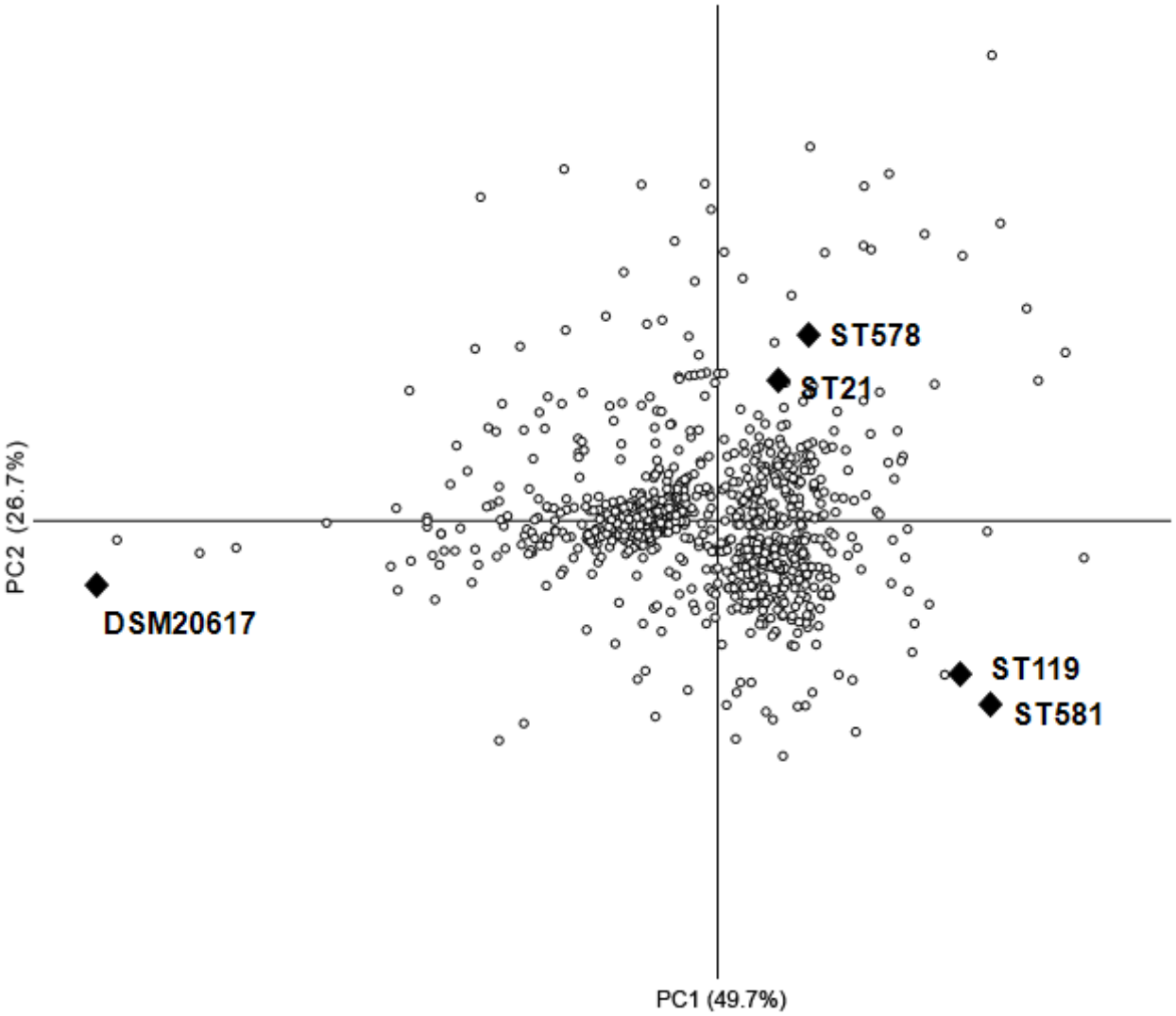

Fig. 4. Principal component analysis of phenotype microarrays profiles of *S. termophilus* strains obtained from an analysis of 960 phenotypic tests (PM11-PM20). The figure shows the entries for five strains (DSM20617, MIM21, MIM119, MIM578, MIM581) and the phenotypical tests (empty circles) plotted in an *X*-*Y* diagram corresponding to the first two components (the BioNumerics Manual version 3.5, Applied Maths).

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