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# Rapid assessment of the physiological status of *Streptococcus macedonicus* by flow cytometry and fluorescence probes

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**Abstract**

Flow cytometry in combination with fluorescence probes was applied to rapidly assess the physiological status of *Streptococcus macedonicus* ACA-DC 198, a newly described member of the lactic acid bacteria group with technologically important features (e.g. lantibiotic production). A sonication procedure was developed for disaggregating typical streptococci chains in order to optimize cell preparations for single cell analysis. Single stained live and dead populations of *S. macedonicus* cells were clearly resolved based on membrane potential by bis-oxonol [$DiBAC_4(3)$], membrane integrity by Propidium Iodide (PI) and enzymatic activity as well as membrane integrity by Carboxyfluorescein Diacetate (cFDA). Further, estimation of both live and dead cells by a cFDA/PI two-colour flow cytometric assay showed excellent correlation with the dead cells in the samples ($dead_{FCM}=0.9945\ dead_S-0.806$, $R^2=0.9986$ and $live_{FCM}=-0.978\ dead_S+98.895$, $R^2=0.9992$). Finally, the assay was applied to study the physiology of *S. macedonicus* after acid stress. Interestingly, *in situ* assessment of the physiological status of stressed *S. macedonicus* cells by flow cytometry and single cell sorting revealed the coexistence of three distinct subpopulations according to their fluorescence labelling behaviour and culturability, representing intact/culturable, permeabilized/dead and potentially injured cells with the latter exhibiting both metabolic activity and membrane permeabilization as well as decreased culturability.


*Keywords:* *Streptococcus macedonicus*; Flow cytometry; Bis-oxonol; Carboxyfluorescein diacetate; Propidium iodide; Single cell; Heterogeneity; Stress

## 1. Introduction

The functional status (viability) of bacterial populations is a critical piece of information under different experimental contexts of microbiology like bacterial stress physiology, antibiotic resistance, environmental sampling etc. Culture based techniques that rely on the propagation of the bacterial cells in an appropriate medium under constant conditions are used as reference methods. Amongst the advantages of these techniques are high-resolution limits and low cost deriving from the fact that neither sophisticated equipment nor specialized staff is required.

However, clonal populations have been shown to exhibit a high degree of heterogeneity in both yeast (Sumner and Avery, 2002) and bacteria (Davey and Kell, 1996). Factors contributing to the appearance of diverse subpopulations within an expected homogeneous population are spontaneous mutations, asynchronous progression through cell cycle, ageing, difference in physiological status and stochastic variations (Booth, 2002; Brehm-Stecher and Johnson, 2004). When survival of a bacterial population after exposure to an environmental insult is assessed by classical Colony Forming Units (CFUs), a binary logic is imposed for the determination of viability. Live cells will be those that managed to replicate under the particular experimental conditions, while all the others will be presumed dead (Kell et al., 1998). Nowadays, it is well documented that under such conditions a population will exhibit cell subpopulations with phenotypes that most likely escape this two-value logic. Injured, viable but not culturable (or active but not culturable as otherwise stated), dormant cells etc. cannot be easily traced or even detected by traditional CFUs (Kell

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et al., 1998; Kell and Young, 2000). Additionally, attempts to circumvent the length of time to yield results with plate counts (24 h to 1–2 weeks for a colony to become visible) with bulk measurements like optical density (OD), respiration level and ATP concentration to assess viability in most cases failed (Davey and Kell, 1996). Finally, one of the major drawbacks of CFUs that is particularly relevant with this report is the inability to determine whether any colony formed on a plate derived from a single cell or a clump of cells (Breeuwer and Abee, 2000; Nebe-von-Caron et al., 2000). Streptococci exhibit a planar division with cells remaining attached to each other, thus, producing chains characteristic for the genus. Survival of cells after a perturbation expressed as a decline in numbers of CFUs is most probably an erroneous quantification of the real number of cells that survived or died. This is due to the distribution of dead cells in chains with live cells that will ultimately give rise to colonies (Braga et al., 2003).

For all reasons mentioned above, fluorescence techniques combined with direct optical detection methods for the rapid assessment of bacterial viability are increasingly being favoured (Bunthof et al., 2001; Ueckert et al., 1995). They are highly sensitive and have high time resolution (Breeuwer and Abee, 2000). Analysis can be performed by epifluorescence or confocal microscopy, scanning or image cytometry as well as flow cytometry. In any of these approaches, measurements can be made at the single cell level. The combination of single cell with multicolour analysis, that is, assessment of different cellular characteristics after simultaneous probing with different fluorochromes, facilitates the detailed unravelling of heterogeneity, a fundamental advantage over conventional methods (Shapiro, 2000b). Particularly, flow cytometry has been shown to be a powerful tool for analysing populations rapidly on a cell-by-cell basis (hundreds to 25,000 cells/s) thus allowing multi-sample processing, whereas in cases of large cell number acquisitions, even rare events can be easily studied with statistical significance (Davey, 2002; Nebe-von-Caron et al., 2000). When fluorescence labelling of the cells is rapid, flow cytometry facilitates near real time monitoring, which is a very important feature for many industrial processes (Hewitt and Nebe-Von-Caron, 2001).

We recently described the new species *S. macedonicus*, within the genus *Streptococcus*, isolated from the flora of naturally fermented Greek Kasseri cheese (Tsakalidou et al., 1998). A first screening of *S. macedonicus* properties revealed strains with technologically important features, such as the production of a lantibiotic exhibiting antimicrobial activity against *Bacillus cereus*, *Clostridium sporogenes*, *Cl. tyrobutiricum*, *Listeria innocua* and *L. ivanovii* (Georgalaki et al., 2002). Macedocin, as the bacteriocin peptide was named, is a promising biopreservative due to its broad antimicrobial spectrum (Bonn, 2003). This attribute makes *S. macedonicus* a perfect candidate to be used as a protective culture since practical applications of LAB aim to take advantage of their potential to improve food hygiene and to assure food quality (Holzapfel et al., 1995). However, rational selection of a strain as a starter or adjunct starter culture requires prior knowledge of its basic physiology that will elucidate whether it will be able to withstand and perform under harsh environments encountered during food processing and storage (van de Guchte et al., 2002). Before attempting to simulate the complexity of the multi-stress conditions imposed during industrial processes, the fundamental features of *S. macedonicus* behaviour under stressful conditions should be explored. For this, an accurate flow cytometric assay that rapidly evaluated the live/dead status of cells was developed. To the best of our knowledge this is one of the very few reports concerning the assessment of the viability of a streptococcal strain at the single cell level (Braga et al., 2003; Nebe-von-Caron et al., 2000).

## 2. Materials and methods

### 2.1. Strain and culture conditions

The Gram-positive bacterium *Streptococcus macedonicus* ACA-DC 198, isolated from traditional Greek Kasseri cheese, was routinely stored in MRS medium (Oxoid Ltd., Basingstoke, Hampshire, UK) supplemented with 1.5% agar. Plates were kept at 4 °C for no more than 2 weeks. A single colony picked from the Petri dish was grown overnight in 5 ml MRS broth at 37 °C. 50 μl of this culture were used to inoculate a 5 ml MRS broth culture, from which the mid-log phase cells derived after incubation for 5 h at 37 °C (OD ~ 0.6–0.7).

### 2.2. Disaggregating S. macedonicus chains into single cells

A sonication procedure was developed in order to disaggregate *S. macedonicus* bacterial chains into free single cells. 100 μl of a mid-log phase culture (~ $10^8$ cells) were added to 400 μl of Phosphate Buffer Saline (PBS) pH 7.0 in eppendorf tubes. Samples were processed in a sonicator bath (Transsonic T460, Elma GmbH, Singen, Germany) filled with water at room temperature. Sonication was performed for 1, 2, 3, 4 and 5 min with 1 min intervals and the effect was evaluated microscopically. In order to assess the efficiency of disaggregation of *S. macedonicus* cell chains after sonication, 100 μl were removed aseptically from the samples and serial dilutions were performed in 900 μl buffered peptone water, pH 7.2 (Merck, Darmstadt, Germany). Plating was performed in duplicates on MRS medium supplemented with 1.5% agar and CFUs were counted after 24 h incubation at 37 °C. At least three independent experiments were performed to confirm reproducibility.

### 2.3. Staining procedures

All chemicals were purchased from Sigma (St. Louis, MO, USA). A 1 mM stock solution of $DiBAC_4(3)$ in dimethyl sulfoxide was prepared and stored at −20 °C, while a 250 μM solution in ethanol was stored at 4 °C and served as working solution. A 10 mM cFDA stock solution in acetone was kept at −20 °C, while the working solution was prepared by further dilution in acetone to a final concentration of 1 mM. PI was stored as 1 mg/ml working solution in distilled water at 4 °C. For $DiBAC_4(3)$ staining, 1 ml of mid-log phase culture was resuspended in equal volume of PBS buffer pH 7.0 and was probed with 0.5 μM $DiBAC_4(3)$ for 30 min at 37 °C. Control depolarized mid-log phase cells were prepared by the addition of 10 μM