Journal of Microbiological Methods 85 (2011) 92–97

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

High-throughput screening of microbial adaptation to environmental stress

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ARTICLE INFO

Article history:
Received 23 November 2010
Received in revised form 30 January 2011
Accepted 30 January 2011
Available online 24 February 2011

Keywords:
Actinobacteria
Frankia
Heavy metals
Screening
Tetrazolium salt

ABSTRACT

We developed a microwell plate, high-throughput, screening method aimed at quantitating the tolerance of a panel of Gram-positive and Gram-negative bacteria to metals (*Frankia* sp., *Escherichia coli*, *Cupriavidus metallidurans*, *Rhizobium leguminosarum*, and *Streptomyces scabies*). Microbial viability was quantified using MTS; a tetrazolium salt converted to a water-soluble formazan through microbial reduction. In this paper, we present the stepwise development of the method, highlighting the main elements underlying its reliability, and compare results obtained with literature. We conclude the method is well suited to efficiently screen bacteria, including those that are filamentous and slow-growing, without the need for large amounts of inoculum which may not always be available. The method allows testing of compound gradients with sufficient replicates to generate statistically robust results, and is transposable to other types of cell proliferation assays such as those for antimicrobial susceptibility, and chemoresistance.

1. Introduction

Adaptation to environmental change is essential for microbial survival and proliferation. Environmental changes will shift the balance of complex microbial communities by favoring some populations, while restricting others, through mechanisms such as microbial competition (for nutrients), antibiosis and by the selection of those microorganisms best adapted to environmental stress. Thus, much research in microbial ecology focuses on the evolution of microbial populations (both taxonomic and functional groups) submitted to fluctuations in environmental conditions (Amoroso et al., 1998; Baath et al., 1998; Baek and Kim, 2009; Baek et al., 2009; Carrasco et al., 2005; Castro et al., 1997; Raniard et al., 2008).

One bacterial genus of interest is that of *Frankia*. *Frankia* are filamentous actinobacteria capable of fixing dinitrogen as soil saprophytes, or while in symbiosis with actinorhizal plants. It is estimated that frankiae and their host plants are responsible for as much as 15%–25% of global nitrogen fixation (Dawson, 2008). In essence, *Frankia* spp. are environmentally relevant bacteria whose potential could be exploited in revegetation and rehabilitation of disturbed ecosystems (Roy et al., 2007).

The microtiter plate format developed enables us to test many conditions in parallel and can be performed with limited amounts of microbial biomass which is critical for slow-growing bacteria. Due to *Frankia* spp. filamentous morphology, methods like turbidity measurement or viable and microscopic counts are unsuitable to estimate proliferation or viability. Therefore, in this study, tetrazolium salt reduction was used to quantify the viability (respiration) of frankiae following exposure to gradients of environmental stress. Tetrazolium salts have been used to measure the electron transport and dehydrogenase activity in bacterial community as well as in pure cultures (McCluskey et al., 2005; Mosher et al., 2003; Obbard, 2001; Prin et al., 1990; Tsukatani et al., 2008; Wang et al., 2010). Tetrazolium salts such as 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT) are reduced into an intracellular insoluble formazan that has to be extracted and dissolved into organic solvent prior to spectrophotometric quantification (Prin et al., 1990; Mosher et al., 2003). We have found however that complete extraction of insoluble formazan from *Frankia* spp. hyphae is unfeasible in the microtiter well format without using mechanical lysis (sonication) which is time consuming (unpublished). To overcome this issue the method described here uses MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium, inner salt), a commercially-available tetrazolium salt that produces water-soluble formazan. MTS has been used as an indicator of proliferation or viability for both eukaryotic cells and prokaryotic microorganisms (Dungan and Hamilton, 1995; Ganguly et al., 2006; Goodwin et al., 1995; Leverone et al., 1996).

This method provides an efficient, miniaturized platform that allows the measuring of the microbial component response over a gradient of a particular environmental parameter (e.g. a concentration gradient of heavy metals). Microbial response in the face of such a gradient is critical since it makes possible to rigorously correlate cause and effect, and determine any threshold effects. Unfortunately, and even before considering the necessity of replicates, studying the effect of hundreds of environmental conditions can be fastidious, and sometimes impossible. The following work describes the development of an easy-to-use, quantitative, inexpensive and rapid screening method that resolves this and appears compatible with other

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doi:10.1016/j.mimet.2011.01.028

clinically or environmentally important bacteria. The method is also transposable to screens aimed at determining microbial response in specific media (carbon sources, etc.), tolerance to antimicrobial agents and may be of particular interest for filamentous microorganisms like actinobacteria.

2. Material and methods

2.1. Bacterial strains

Four *Frankia* spp. strains were used throughout the optimization experiments and the screening for metal tolerance. They were *Alnus* isolates ACN10a, ACN12a (Normand and Lalonde, 1982) and AvcI1, and *Casuarina* isolate CcI3. Most of these strains come from the collection of Pr. Maurice Lalonde and were generously provided by the *Centre d'étude de la forêt of Université Laval*, Quebec. All *Frankia* strains were cultivated at 30 °C (static) in BAPS medium (BAP as described by Igual and Dawson (1999), supplemented with 5 g/L sodium succinate). For all experiments, two to four week-old cultures were used as the inoculum. Hyphae were centrifuged at 3440×*g* for 10 min at 4 °C. The pellet was washed twice and resuspended with sterile saline (0.85% w/v NaCl). To increase dispersal and help maintain method reproducibility, suspensions of these filamentous bacteria were homogenized before being transferred to microwell plates. This was accomplished using a 10 cc syringe with an 18 G1½ needle (Echbab et al., 2007; Gabbarini and Wall, 2008; Montpetit and Lalonde, 1988; Nickel et al., 2001; Valverde and Wall, 2005). To standardize microwell plate inocula based on protein concentration, total protein content of these homogenized cell suspensions was determined as follows: three aliquots (sub-samples) were transferred in 2 mL Cryotubes® (Sarstedt, Newton, NC) where 1.3 to 1.5 g of SiLibeads® zirconium–yttrium stabilized beads (Type ZY, 0.6–0.8 mm, Sigmund Linder, Warmensteinach, Germany) were added for the cell disruption process. The samples were lysed with a FastPrep-24 (MP Biomedicals, Solon, OH) for six 15-second treatments at a setting of 6.5 m/s and aliquots were kept on ice for 5 min between each disruption treatment. Total *Frankia* protein concentration was quantified using the Bio-Rad Protein Assay, based on the coomassie Bradford method (Bio-Rad, Hercules, CA).

Other bacterial strains studied were *Escherichia coli* K12 (ATCC 10798) cultivated in BAPS medium, *Cupriavidus metallidurans* (ATCC 43123) cultivated in nutrient broth (Difco Laboratories, 1998), *Rhizobium leguminosarum* bv. *viciae* strains Lj3, Lj8 and Lp0610 cultivated in YMB medium (Drouin, 1997), and *Streptomyces scabies* strain EF-35 cultivated in YME (Beauséjour and Beaulieu, 2004). The same media were used in the determination of the strains resistance to metals. All these strains were cultivated with 250 rpm agitation, and *E. coli* K12 was cultivated at 37 °C while *C. metallidurans*, *R. Leguminosarum* bv. *viciae* and *S. scabies* EF-35 were cultivated at 30 °C. Inoculum preparation and total protein determinations for these microorganisms prior to microwell plate inoculation were done as described above for *Frankia* strains.

2.2. Microwell plate preparation and incubation

Microwell plates used were standard 96-well plates (Falcon 3072, Fisher Scientific, Whitby, ON) (Roy et al., 2005). All microwell plates were prepared with 2X BAPS medium (for frankiae) or the suitable media for other microorganisms. Each well contained 100 µL of 2X culture medium spiked with 50 µL of the contaminant of interest (for the complete ranges tested, see Table A.1), and then 50 µL of inoculum was added, bringing the total volume to 200 µL. Final *Frankia* protein concentrations of 10, 15, 20, 25, 30 and 35 µg/mL were assayed in order to determine which would give an adequate signal as described in Section 3.1.1. Microwell plates were sealed with Parafilm and incubated for 2 weeks in closed, 10-L polypropylene containers, at room temperature (RT) and protected from light. A beaker containing sterile water was placed in these containers to limit "edge effect" evaporation during incubation. For *E. coli* K12, microwell plate incubation lasted 20 h at 37 °C, for *C. metallidurans*, *S. scabies* and *R. leguminosarum* bv. *viciae*, incubation lasted 48 h at 30 °C.

2.3. Revelation of microbial viability using MTS/PMS coloration

MTS reagent powder (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium, Promega, Madison, WI) and PMS (phenazine methosulfate, Sigma, St-Louis, MO) were dissolved in 0.85% w/v NaCl at concentrations of 2 mg/mL and 0.046 mg/mL, respectively, as recommended by the supplier. The solution was filter-sterilized with 0.22 µm filters and stored at −20 °C protected from light. MTS/PMS solution was freshly thawed before use in microwell plates. The volume of MTS/PMS solution added to each well (5, 10, 15, 20 and 40 µL) was optimized during this study, as described in Section 3.1.2. After the addition of MTS/PMS in culture media, microwell plates were kept at 30 °C for various time periods (2, 4, 8, 16 and 24 h) as described in Section 3.1.3. Microwell plates were then centrifuged 5 min at 2634×*g* to precipitate *Frankia* spp. aggregates. Then, 100 µL supernatant from each well were transferred to a new microwell plate. This last plate was read at 490 nm using an Asys model UVM 340 microwell plate reader (Biochrom, Cambridge, UK).

For all microorganisms tested in this study, final protocol parameters were an inoculum concentration of 15 µg/mL, 15 µL of MTS/PMS, and a 4-hour incubation time before spectrophotometric quantification of reduced MTS reagent.

3. Results and discussion

3.1. Method development using Frankia spp

3.1.1. Optimization of inoculation density

Proliferation as a function of inoculum density was studied since many toxic compounds target specific bacterial mechanisms activated during multiplication (e.g. translation or respiratory metabolism) (Behal, 2006; Zhou et al., 2005). In addition, *Frankia spp.* are difficult to grow in general, and when inoculum density is too low, it may take time to proliferate or strains simply will not grow at all (personal observations). As shown in Fig. 1, microwell plates were inoculated at different final inoculum protein concentrations using two *Frankia* strains, and then incubated either 1 day or 2 weeks. Following this short or long incubation, 15 µL of MTS/PMS per well were added to measure microbial viability. The 1 day incubation was done to determine the basal respiration level of the strains and the 2 weeks incubation simulated a screening test that included potential proliferation and was of sufficient length to allow *Frankia* proliferation. After 1 day of incubation, MTS reduction was positively correlated with the initial amount of inoculum (protein concentration) for all strains. However, after 2 weeks of incubation, the results between strains varied. Strain ACN10a reduction of MTS was still positively correlated with initial inoculum (protein) concentration. This could be explained by the fact that this strain did not proliferate rapidly and that all wells still contained a different quantity of *Frankia*. Since each hyphae can reduce a given amount of MTS, the amount of formazan formed would be dependent on the quantity of mycelium. For strain AvcI1, MTS reduction was quite constant independently of the initial protein concentration of the inoculum (10 to 35 µg/mL).

To exclude the possibility that all the MTS present in the medium had been reduced into its formazan form and that it was in fact a limiting factor (an unwanted outcome), a second experiment was performed. A microwell plate was prepared and inoculated with *Frankia* sp. at two different initial concentrations (15 and 30 µg/mL). Microwell plates were then incubated for 2 weeks to reproduce