Note

Modified in situ antimicrobial susceptibility testing method based on cyanobacteria chlorophyll *a* fluorescence

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A B S T R A C T

The chlorophyll *a* fluorescence based antimicrobial susceptibility testing (AST) method presented in a previous work was based on the measurement of Chl *a* fluorescence of the gram(−) cyanobacterium *Synechococcus* sp. PCC 7942. *Synechococcus* sp. PCC 7942 as a gram(−) bacterium is affected by antibacterial agents via mechanisms affecting all gram(−) bacteria, however, as an exclusively phototrophic organism it would also be affected by photosynthesis inhibitory action of an agent that otherwise has no antibacterial properties. In this report, the method is modified by replacing the exclusively phototrophic *Synechococcus* sp. PCC 7942 with the *Synechocystis* sp. PCC 6714, capable of both phototrophic and heterotrophic growth in order to add versatility and better reflect the antibacterial effects of surfaces under study towards nonphotosynthetic bacteria.

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1. Introduction

An increasing focus on health and safety has introduced the need for microorganism population control in several sectors of the everyday life, intensifying research on materials with antibacterial properties. The widespread use of such materials requires an accurate, easy and cost effective way to determine and quantify their antibacterial behavior. Agar based standard tests have been the most frequently applied for that purpose. Recently, a new method based on fluorescent bacteria was introduced as a cheap, easy, fast and accurate alternative to the existing standard antimicrobial susceptibility testing (AST) methods (Heliopoulos et al., 2015a, 2015b). The test organism proposed was the gram(−) cyanobacterium *Synechococcus* sp. PCC 7942 (hereafter *S*.7942), and measurement was based on the emission of Chl *a* fluorescence of the bacteria. *S*.7942 as a gram(−) bacterium is affected by antibacterial agents via mechanisms affecting all gram(−) bacteria, however, as an exclusively phototrophic organism it would also be affected by photosynthesis inhibitory action of an agent that otherwise has no antibacterial properties.

In that sense the exclusively phototrophic nature of the test organism could be proven a shortcoming when testing materials without antibacterial properties but with photosynthesis inhibition properties, where a false positive result would be obtained.

In this report, the method is modified by replacing the exclusively phototrophic *S*.7942 with the *Synechocystis* sp. PCC 6714 (hereafter *S*.6714), capable of both phototrophic growth by oxygenic photosynthesis and heterotrophic growth by glycolysis and oxidative phosphorylation (Heidorn et al., 2011). A case study is also presented with its application on wool fabric materials with antibacterial properties tested in a previous work (Heliopoulos et al., 2013) to allow for direct comparison of the results.

2. Materials and methods

For the antimicrobial finishing commercial undyed 100% wool fabric (weight, 155 g/m^2) with plain weave was used. Medium viscosity (200 cP) sodium–alginate, average molecular weight 6000, Mannuronic/ Guluronic acid ratio 1.75 ± 0.12, and $Cu(NO_3)_2 \cdot 5H_2O$ were purchased from Sigma-Aldrich. Levantin LNB was purchased from BASF. 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea (DCMU) was purchased from Sigma-Aldrich. Acetic acid 100% was purchased from Merck and Tetracloroethylene from Panreac.

The unicellular cyanobacterium *Synechocystis* sp. PCC 6714, obtained from the Collection Nationale de Cultures de Microorganismes CNCM, Institut Pasteur, Paris, France, was used in all experiments.

All chemicals were of analytical grade and were used without any further purification.

3. Preparation of wool fabrics

The samples to be tested were prepared as described previously (Heliopoulos et al., 2013). In detail, wool fabric was cleaned in a bath containing 1.0% non-ionic washing agent Levantin LNB at a liquor-to-

fabric ratio of 30:1 for 15 min at 40 °C. The pH was adjusted at 4.5 by addition of acetic acid solution (10 g/L). The fabric was subsequently rinsed with warm bi-distilled water (40 °C) for 3 min and then with cold bi-distilled water (25 °C) for 9 min. The samples were then dried at room temperature.

For the preparation of wool/copper fabrics (WCF), the washed wool specimens were immersed under agitation in an orbital shaker at 180 rpm in aqueous solutions of 5000 mg/L $Cu(NO_3)_2 \cdot 5H_2O$ at 25 °C for 24 h, at a liquor-to-fabric ratio 30:1. Finally, the samples were rinsed with cold bi-distilled water and dried at room temperature. The wool/alginate/copper fabrics (WACF), were prepared as follows. Alginate solution was prepared by dissolving sodium alginate powder in bi-distilled water at a concentration of 2.0% (w/v) in which pre-weighed wool fabric samples, cut in sizes of around 20×12 cm, were immersed for 15 min at room temperature, squeezed to 100% wet pick-up on a laboratory padding mangle and dried at room temperature. Treatment with alginate resulted in a wool/alginate sample containing $\approx 2\%$ w/w alginate.

Finally, the wool–alginate samples were immersed under agitation in an orbital shaker at 180 rpm in aqueous solutions of 5000 mg/L $Cu(NO_3)_2 \cdot 5H_2O$ at 25 °C for 24 h, at a liquor-to-fabric ratio 30:1, for the preparation of a Cu/alginate coating. The samples were rinsed with cold bi-distilled water and dried at room temperature.

The total content of Cu in the wool and wool–alginate fabrics was quantitatively determined by measuring the remaining copper concentration on the liquid using a GBC GF 300 Avanta atomic absorption spectrometer (AAS).

The Cu^{2+} solution treatment, yielded samples containing ≈ 6.0 mg/g Cu^{2+} for the wool/copper fabric (WCF) and ≈ 10.0 mg/g Cu^{2+} for the wool/alginate/copper fabric (WACF) a Cu^{2+} content difference due to the superior sorption capacity of alginate that can hold up to more than 172.0 mg/g of copper ions (Deze et al., 2012).

4. Washing treatment

Two washing procedures were used to challenge the durability of the antimicrobial activity of WCF and WACF upon repeated laundering.

According to the first procedure (BS EN ISO 105-D01), samples were washed in a Rotawash M228-SDL International machine with tetrachloroethylene without other solvents. The procedure was repeated 5 times.

Additionally, fabric samples were washed with liquid carbon dioxide as an alternative to potentially harmful solvents such as tetrachloroethylene used in dry cleaning. Samples were soaked in liquid CO_2 at room temperature and were kept under constant CO_2 flow of 0.8 mL/min in an open flow system at 100 bar pressure for 25 h. The total liquid CO_2 volume used was 1200 mL. CO_2 washing was equivalent to fifty 30-min washings.

5. AST

S.6714 cells were cultured in BG11 (Rippka et al., 1979) that contained additionally 20 mM HEPES/NaOH, pH 7.5 (basal medium). The cultures were incubated under white fluorescent light ($100 \, \mu E \cdot m^{-2} \cdot s^{-1}$), in an orbital incubator (Galenkamp INR-400) at 31 °C (Stamatakis and Papageorgiou, 2001). Culture growth was monitored in terms of concentration of Chl a, determined in N,N-dimethylformamide extracts of cell pellets (Moran, 1982).

For the fabric surfaces testing, suitable quantity of S.6714 cells was harvested from the culture suspensions by centrifugation (5000 rpm, 5 min) and was resuspended in buffered BG11, so that the concentration of Chl a was $52.0 \, \mu g \cdot mL^{-1}$. Two identical S.6714 batches were prepared. In the first batch 20 μM DCMU was added and in the second a combination of 20 μM DCMU/5 mM Glucose. A drop of the abovementioned solution was pipetted (0.05 mL) on each test specimen producing a stain of less than 3.0 mm diameter on the fabric.

Samples were dark-adapted for 15 min in a suitable clip and Chl a fluorescence was measured using a PEA-fluorometer (PEA, Hansatech Instruments LTD, Norfolk, UK) Cyanobacteria growth and growth inhibition on wool samples were monitored by measurement of the Chl a fluorescence every 24 h for seven days. Throughout the course of seven days the samples were kept in an incubator under white fluorescent light ($100 \, \mu E \cdot m^{-2} \cdot s^{-1}$) at 31 °C. The F_0 value of the cyanobacterial colony stain on each wool sample was recorded every 24 h for a period of seven days and the corresponding M_ι as well as the corresponding Π_7 values were calculated according to Heliopoulos et al. (2015a, 2015b).

Specifically, the % change (M_ι) in Chl a fluorescence after i days of incubation is calculated by Eq. (2)

$$M_\iota = \frac{F_{0_i} - F_{0_0}}{F_{0_0}} \times 100 \qquad (1)$$

where F_{0_0} is the value of Chl α fluorescence of cyanobacterium at zero contact time and F_{0_i} is the value of Chl α fluorescence of cyanobacterium after 1,2,...,i days.

The material's antibacterial action is represented by the Bacterial Protection Index (BPI) Π_7, given by the equation:

$$\Pi_7 = \frac{M_{U_7} - M_{T_7}}{M_{U_7}} \times 100 \qquad (2)$$

where M_{U_7}, is the change in cyanobacterial Chl α F_0 value on the untreated sample after seven days of incubation and M_{T_7}, is the change in cyanobacterial Chl α F_0 value on the treated sample after seven days of incubation.

All experiments were performed in three replicates using different S.6714 cell cultures.

6. Results and discussion

6.1. Antimicrobial susceptibility testing

In order to evaluate S.6714 different metabolic pathways and assess its applicability to the proposed AST method, cell proliferation was monitored in liquid cultures of S.6714 cyanobacteria, by measurements of Chl a concentration in the course of five days, either after addition of 20 μM DCMU or 20 μM DCMU/5 mM Glucose in the BG11/HEPES/NaOH culture medium and the results are presented in Fig. 1.

As expected, when under conditions of suppression of both modes (BG11/20 μM DCMU), cell population is decreasing to approach zero after 5 days. In heterotrophic (BG11/20 μM DCMU/5 mM Glucose) or phototrophic (BG11) modes cell growth follows almost the same
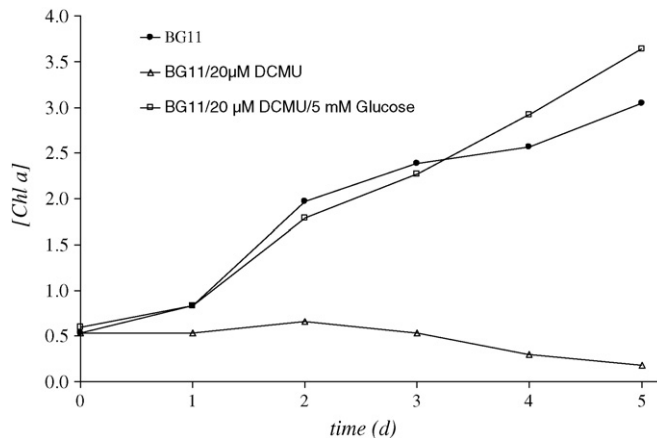

Fig. 1. Cell proliferation curves of Synechocystis sp. PCC 6714 in BG 11 (control cells;—■—), in the presence of 20 μM DCMU (—△—), in the presence of 20 μM DCMU and 5 mM Glucose (—□—).