Resistance of Pseudomonas aeruginosa biofilms to cold atmospheric pressure plasma treatment is linked to the redox-active molecule phenazine

A. Mai-Prochnow¹, M. Bradbury², K. Ostrikov¹,³ and A.B. Murphy¹

¹ CSIRO Manufacturing Flagship, Sydney, NSW, Australia
² CSIRO Food and Nutrition Flagship, Sydney, NSW, Australia
³ Institute for Health and Biomedical Innovation, School of Chemistry, Physics and Earth Sciences, Queensland University of Technology, Brisbane, QLD, Australia

Abstract: In this study we report on significant advances in understanding the response of biofilms of P. aeruginosa to cold atmospheric plasma (CAP). Our study shows that CAP has the potential to eradicate antimicrobial-resistant biofilm bacteria. We also discover that low doses may lead to the surviving bacteria being more resistant to subsequent plasma exposure. We provide strong evidence that the resistance is associated with production of the redox-active pigment phenazine by the bacteria.

Keywords: Cold plasma, Biofilm, Pseudomonas, Resistance

1. Introduction

A number of studies show very promising results for cold atmospheric plasma (CAP)-mediated killing and removal of biofilms, including pathogenic bacteria such as Pseudomonas aeruginosa [1, 2]. Biofilms are the predominant mode of growth for bacteria. They are cell clusters encapsulated by an extracellular matrix attached to a surface. It is now widely recognized that more than 60% of all infections are caused by biofilm-forming bacteria. These biofilm infections can become resistant to treatment with traditional antibiotics and often develop into a chronic state.

2. CAP effect on biofilms

Biofilms were grown in a CDC biofilm reactor [3] and treated using the kINPen med (Neoplas tools GmbH, Greifswald, Germany) [4]. The kINPen hand-held nozzle was connected to a base unit with a gas feeding bottle. Argon was used as a feeding gas and plasma pulses are generated at a frequency of 1.82 MHz at a gas flow rate of 4.2 slm. After a range of treatment times (1, 3, 5 and 10 min), biofilms were subjected to viable cell counts (Table 1) and confocal microscopy (Fig. 1) to investigate the effect of CAP on biofilms. The untreated control biofilms reached cell numbers of 4.5 x 10⁵ CFU per ml (Table 1). The gas control showed a slight decrease in CFU numbers (3.9 x 10⁴ per ml), possibly due to a drying effect from the gas flow. After only 1 min of plasma treatment, microcolonies were significantly smaller and some dead cells appeared. Large microcolonies are removed after 3 min of plasma treatment with most of the biofilm removed after 5 min and no cells left after 10 min (Fig. 1).

Table 1. CFU counts of P. aeruginosa biofilms cells after argon plasma treatment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CFU counts (ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>4.5 (± 0.6) x 10⁵</td>
</tr>
<tr>
<td>Gas control</td>
<td>3.9 (± 0.6) x 10⁴</td>
</tr>
<tr>
<td>1 min CAP</td>
<td>1.8 (± 0.3) x 10⁴</td>
</tr>
<tr>
<td>3 min CAP</td>
<td>2.1 (± 0.4) x 10⁴</td>
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<tr>
<td>5 min CAP</td>
<td>*</td>
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<tr>
<td>10 min CAP</td>
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* indicates counts were below detection limit (1 x 10²)

In addition to CFU numbers, a second set of biofilms grown on glass coupons was observed with confocal laser scanning microscopy. The untreated control showed attached, viable cells forming microcolonies approximately 10 µm thick (Fig. 1A). The gas control showed similar size microcolonies. A few single dead cells were observed in the gas control, presumably due to a drying effect from the gas flow. After only 1 min of plasma treatment, microcolonies were significantly smaller and some dead cells appeared. Large microcolonies are removed after only 3 min of plasma treatment with most of the biofilm removed after 5 min and no cells left after 10 min (Fig. 1).

3. Bacterial resistance to CAP

To investigate a possible bacterial resistance mechanism to plasma treatment, a lawn of P. aeruginosa cells was spread onto agar plates and exposed to argon plasma treatment. Surviving bacterial colonies from plasma and gas control plates were selected for whole genome sequencing. Comparison between surviving cells and control cells revealed 10 distinct polymorphic regions, including four belonging to the redox-active,
antibiotic pigment phenazine. Subsequently, the interaction between phenazine production and CAP resistance was demonstrated in biofilms of transposon mutants disrupted in different phenazine pathway genes, which exhibited significantly altered sensitivity to CAP (Fig. 2). Mutants whose ability to produce phenazine is compromised (ΔphzD, ΔphzE and ΔphzF) show significantly reduced survival under CAP exposure.

4. Discussion
Pathogenic bacteria exhibiting resistance to antibiotic agents have become an area of great concern. Whilst antibiotic resistance in bacteria occurs naturally to some extent [5, 6], inappropriate use and overuse of these drugs has increased this process at an alarming rate [7]. In particular biofilms, which account for over 60% of infections, show a high degree of resistance [8].

Results of our study clearly demonstrate that *P. aeruginosa* biofilms of about 15 µm thick can be completely eradicated using a 10 min plasma treatment with a kINPen med. However, a low dose (3 min) plasma treatment allowed for some cells to survive, which subsequently exhibited a higher resistance to further plasma treatment. DNA sequencing revealed that this higher resistance is related to mutations in phenazine biosynthesis genes. Phenazines were previously shown to increase resistance to antibiotics [9] and promote anaerobic survival via extracellular electron transfer [10], and are involved in protection to environmental oxidative stress [11]. To our knowledge this is the first report describing a role for phenazines in the response to CAP.

5. Acknowledgements
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6. References