Increased mutability of *Pseudomonas aeruginosa* in biofilms


Antimicrobial Research Centre and Institute of Molecular and Cellular Biology, University of Leeds, Leeds LS2 9JT, UK

Received 21 November 2007; returned 4 January 2008; revised 15 January 2008; accepted 16 January 2008

**Objectives:** Isolates of *Pseudomonas aeruginosa* from cystic fibrosis (CF) patients are frequently hypermutable due to selection of mutants with defects in DNA repair genes such as *mutS*. Since *P. aeruginosa* grows as a biofilm within the infected CF lung, it is possible that this mode of growth enhances the mutability of the organism thereby increasing the opportunity to derive permanent hypermutators through mutation in DNA repair genes. We have now conducted experiments to examine this possibility.

**Methods:** Using established procedures, we examined the mutability of *P. aeruginosa* PA01 in planktonic cultures and in biofilm cultures generated by growth in a Sorbarod system. Transcriptional profiling by DNA microarray was used to compare gene expression in planktonic and biofilm cells.

**Results:** Mutation frequency determinations for resistance to rifampicin and ciprofloxacin demonstrated that biofilm cultures of *P. aeruginosa* displayed up to a 105-fold increase in mutability compared with planktonic cultures. Several genes (*ahpC*, *katA*, *sodB* and PA3529, a probable peroxidase) that encode enzymes conferring protection against oxidative DNA damage were down-regulated in biofilm cells. In particular, *katA*, which encodes the major pseudomonal antioxidant catalase, was down-regulated 7.7-fold.

**Conclusions:** Down-regulation of antioxidant enzymes in *P. aeruginosa* biofilms may enhance the rate of mutagenic events due to the accumulation of DNA damage. Since *P. aeruginosa* forms biofilms in the CF lung, this mode of growth may enhance the direct selection of antibiotic-resistant organisms in CF patients and also increase the opportunity to derive permanent hypermutators thereby providing a further source of antibiotic-resistant mutants in the CF lung.

Keywords: mutation frequencies, microarrays, antibiotic resistance, hypermutators

**Introduction**

Hypermutable strains of *Pseudomonas aeruginosa*, with defects in the methyl-directed mismatch repair (MMR) system, are frequently isolated from the lungs of cystic fibrosis (CF) patients. In contrast, such strains are not readily recovered from other patients infected with this opportunistic pathogen. A strong relationship between antibiotic resistance and hypermutability has been observed in *P. aeruginosa* isolates from the CF lung, suggesting that hypermutability confers a selective advantage for the pathogen in this environment where the organism is often exposed to high doses of antibiotics for prolonged periods as part of the therapeutic regimen for CF patients.

Although the CF lung appears to provide an environment that favours the development and selection of hypermutators in *P. aeruginosa*, the factors responsible are poorly understood. Infection of the CF lung results in a chronic inflammatory response with liberation of reactive oxygen species (ROS), which are capable of causing DNA damage and mutagenesis. Indeed, ROS-mediated mutagenesis has been implicated as a factor in the emergence of antibiotic-resistant hypermutable *P. aeruginosa* during infection of CF patients. However, in the CF lung, *P. aeruginosa* grows as a biofilm which is poorly penetrated by ROS. Consequently, it is possible that the mutagenic effect of ROS on *P. aeruginosa* in the CF lung is enhanced by the physiological status of the organism itself. We have now examined whether there is a relationship between the mutability of *P. aeruginosa* growing in biofilms and expression of genes involved in protection against ROS-mediated DNA damage, or its repair.

*Corresponding author. Tel: +44-113-343-5604; Fax: +44-113-343-1407; E-mail: i.chopra@leeds.ac.uk
†Present address. Smith and Nephew Research Centre, York Science Park, Heslington, York YO10 5DF, UK.

© The Author 2008. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oxfordjournals.org
Materials and methods

Bacteria, growth media and chemicals

P. aeruginosa PA01\textsuperscript{9} was obtained from Dr Raphael Canton, Hospital Universitario Ramón y Cajal, Madrid, Spain. This strain was routinely cultured at 37°C in Iso-Sensitest broth (ISB) or ISO-Sensitest agar (ISA), which were purchased from Oxoid (Basingstoke, UK). All chemicals, reagents and antibiotics were purchased from Sigma (Poole, UK) with the exception of ciprofloxacin, which was a gift from Bayer AG (Leverkusen, Germany).

Antibacterial susceptibility testing

MIC determinations were performed on planktonic cultures of P. aeruginosa PA01 by agar dilution, using inocula of 10\textsuperscript{8} cfu/spot. MICs were defined as the lowest antibiotic concentration preventing visible bacterial growth after 18 h of incubation at 37°C.

Biofilm culture

P. aeruginosa biofilms were created using a modified Sorbarod apparatus.\textsuperscript{10–12} Sorbarod filters (Ilacon Ltd, Tonbridge, UK) were inoculated with 0.5 mL saturated bacterial cultures in ISB (∼10\textsuperscript{9} organisms/mL) and then perfused with ISB at a flow rate of 1 mL/min for 72 h at 37°C. Bacteria were harvested from Sorbarod filters by splitting the paper sleeve to release the fibres, followed by vigorous vortexing (30 s), sonication (5 min) in an ultrasonic water bath and further vortexing (30 s).

Determination of mutation frequencies for resistance to antibiotics

Mutation frequencies for resistance to rifampicin and ciprofloxacin were determined for planktonic and biofilm cultures. Bacterial cells were recovered from biofilms as described above and planktonic cultures were grown in the absence of antibiotic for 18 h prior to plating onto ISA selection plates containing antibiotic at 4× MIC to recover resistant mutants. To determine viable counts, aliquots of diluted culture were plated onto non-selective ISA. Colony counts were made after 24 h of incubation at 37°C on non-selective media and after 48 h of incubation on selective media. Mutation frequencies were expressed as the number of antibiotic-resistant mutants recovered as a fraction of the viable count. Where appropriate, cultures were concentrated by centrifugation to facilitate the recovery of resistant mutants.

Analysis of gene expression in planktonic and biofilm cultures by microarray profiling

Biofilm and planktonic P. aeruginosa cells were collected in RNAProtect (Qiagen, Crawley, UK), and RNA purified using the RNeasy mini kit (Qiagen) according to the manufacturer’s instructions. RNA was submitted to GRI Ltd (Essex, UK) for labelling, hybridization and quantification of gene expression using a NimbleGen microarray and ArrayStar\textsuperscript{™} FirstLight software (DNASTAR). The NimbleGen array contained 5567 of the predicted 5570 open reading frames (ORFs) of P. aeruginosa PA01.\textsuperscript{9} Genes were considered to be differentially expressed in the biofilm if they exhibited a 2-fold increase or decrease in expression compared with planktonic cultures.\textsuperscript{13,14}

Results

Growth of P. aeruginosa in biofilms results in elevated mutation frequencies

The frequency of selection of rifampicin- and ciprofloxacin-resistant mutants of P. aeruginosa PA01 increased ∼15- and 105-fold, respectively, when the organism was grown as a biofilm (Table 1). No hypermutators, defined here as strains with permanent mutation frequencies elevated 10-fold or greater for resistance to two or more antibiotics, were observed among the resistant mutants recovered (data not shown). This suggests that the increased mutability observed in biofilm cultures was predominantly phenotypic rather than genotypic.

Comparison of gene expression in P. aeruginosa planktonic and biofilm cultures

To investigate further the enhanced mutability of biofilm cultures of P. aeruginosa (Table 1), we examined global gene expression profiles by microarray analysis. We were particularly interested in the expression of genes that mediate DNA repair (Table 2) and protect against ROS (Table 3).\textsuperscript{15,16} Genes involved in MMR (mutS, mutL, and uvrD), repair of 8-oxo-dG (GO) lesions (mutM, mutT, mutY and micA) and replication fidelity (mutD and dnaE) were not differentially expressed in the biofilm compared with planktonic cultures (Table 2). Hence, the increased mutability observed in P. aeruginosa PA01 biofilm cells is not due to diminished transcription of these genes. We noted down-regulation of polA expression in biofilm cultures (Table 2). This may contribute to the enhanced mutability of P. aeruginosa in the biofilm (Table 1). Nevertheless, the contribution is likely to be modest since complete elimination of this gene in Escherichia coli only confers a very small increase in mutability.\textsuperscript{17}

However, transcription of several genes involved in defence against ROS\textsuperscript{18} was down-regulated in the biofilm (Table 3). These genes included sodB, katA, ahpC and ORF PA3529, which encodes a probable peroxidase.\textsuperscript{9}

Discussion

Production of antioxidant enzymes and the maintenance of efficient DNA repair systems are first-line defences against oxidative DNA damage in bacteria.\textsuperscript{19} We observed that four genes in P. aeruginosa that encode antioxidant enzymes are

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Mutation frequency\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampicin</td>
<td>2.63 ± 1.79 × 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>1.34 ± 0.89 × 10\textsuperscript{-10}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Mutants were selected on plates containing antimicrobial agents at 4× MIC determined against planktonic cultures.
down-regulated in the biofilm (Table 3). This suggests that the organisms may be particularly susceptible to DNA damage from ROS derived from aerobic respiration. In particular, \( katA \), the primary pseudomonal antioxidant catalase enzyme, was down-regulated 7.7-fold in biofilm cultures when compared with planktonic cultures. It has been demonstrated that KatA is crucial for
oxidative and osmotic protection as well as adaptation to peroxide stress in *P. aeruginosa* in both planktonic cultures and biofilms. In contrast to our findings, a recent transcriptional analysis of gene expression in biofilms of *P. aeruginosa* demonstrated no alteration in expression of katA. The relationship of this observation to our findings is unclear. Unfortunately, the mutational status of the organisms in the biofilm was not determined in the previously published study, which makes further discussion of the apparent discrepancy difficult.

The frequently encountered GO DNA lesion, resulting from ROS attack, is removed and repaired by the GO system. Consequently, we conclude that the biofilm mode of growth adopted by *P. aeruginosa* during infection of the CF lung, in which genes conferring protection against oxidative damage are down-regulated, may increase the mutability of the organism. This creates conditions for the emergence of antibiotic-resistant organisms, e.g. through point mutation in drug targets, and also enhances the opportunity to derive permanent hypermutators through mutation in DNA repair genes such as *mutS*, which is frequently found to be mutated in clinical hypermutator isolates.

**Funding**

The research described here was supported by a BBSRC CASE PhD studentship for K. D. in conjunction with Smith and Nephew Research, York, awarded to I. C.

**Transparency declarations**

None to declare.

**References**


