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Chip-calorimetric monitoring of biofilm eradication with antibiotics provides mechanistic information

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ABSTRACT

Increased antibiotic resistance of pathogenic bacteria dwelling in biofilm structures has motivated the development of various monitoring tools specifically designed for biofilm investigations. In this study, the potential of the recently emerging chip calorimetry for this purpose was analysed. The activity of biofilms of *Pseudomonas putida* PaW340 was monitored chip-calorimetrically and compared with counts of colony forming units (CFU), bioluminescence-based ATP measurements, and quantitative confocal laser scanning microscopy (CLSM). The biofilms were treated with antibiotics differing in their mechanisms of action (bactericidal kanamycin vs. bacteriostatic tetracycline) and referenced to untreated biofilms. For untreated biofilms, all methods gave comparable results. Calorimetric killing curves, however, reflecting metabolic responses to biofilm eradication non-invasively in real time, differed from those obtained with the established methods. For instance, heat signals increased right after addition of the antibiotics. This transient increase of activity was not detected by the other methods, since only calorimetry delivers specific information about the catabolic part of the metabolism. In case of the bactericidal antibiotic, CFU misleadingly indicated successful biofilm eradication, whereas calorimetry revealed enduring activity. Our results show that calorimetry holds promise to provide valuable mechanistic information, thereby complementing other methods of biofilm analysis.

Introduction

A majority of all bacteria in natural and clinical settings live in biofilms, which are surface-associated communities of cells (Costerton et al., 1987, 1995). Undesired effects of biofilms developing on man-made surfaces are referred to as biofouling (De Beer et al., 1994). Even more problematic are biofilms consisting of pathogenic bacteria involved in the development of infectious diseases. For instance, bacterial colonization on indwelling medical devices such as catheters, prosthetic joints, or pacemakers is a major reason of persistent infectious diseases (Hall-Stoodley et al., 2004). Microbial cells associated with biofilms are more difficult to eradicate than their free-floating counterparts. The reasons are (i) the extracellular polymeric substances that act as penetration barrier for antimicrobial agents; (ii) concentration gradients of substrates or products that cause a slowly growing area, and are therefore less prone to most antibiotics; (iii) adaptive stress response; (iv) persistent cells (Stewart, 2002). Furthermore, the horizontal gene transfer is facilitated in biofilms and may help to increase the tolerance of pathogens to antibiotics (Høiby et al., 2010). These characteristics are challenges for the development of new antibiotics or new combinations of antibiotics and also motivate the development of new tools for testing the efficacy of antimicrobial agents and formulations.

Several biofilm methods are simply variants of established methods for planktonic cells. Counting of colony forming units (CFU) growing on agar plates is one of the most frequently applied methods (Rivardo et al., 2011; Kajiyama et al., 2009; Kim et al., 2008; De Beer et al., 1994; Yu and McFeters, 1994; Prosser et al., 1987). It requires invasively disrupting the biofilm structure, and some studies revealed that the number of bacteria is often underestimated by this method (Høiby et al., 2010; Yu and McFeters, 1994; De Beer et al., 1994).

Alternatively, the ATP content in cells is frequently taken as a measure for bioactivity (Kajiyama et al., 2009) or viability (Takahashi et al., 2007) and used to quantify the number of biofilm cells. ATP analysis requires disintegrating cells and

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biofilm structure thereby possibly introducing artifacts (Kajiyama et al., 2009; Monzón et al., 2001), and energy-demanding adaptations to unfavourable growing conditions may bias the results.

Staining cells in biofilms using crystal violet is a well-established method in biofilm analysis (Takahashi et al., 2007). It is quantitative and does not require disruption of the biofilm structure, but the staining procedure is invasive. Moreover, this method has low sensitivity and does not give information about cell viability or activity. Fluorescence-based assays allow for instance to estimate the proportion of 'living' to 'dead' in the biofilm. These methods profit from the development of modern fluorescence microscopy in recent years. They provide insight into the activity distribution in a biofilm structure, but have the disadvantages of high costs and the need to pretreat the biofilm samples (Hannig et al., 2010; Kim et al., 2008).

We are introducing chip calorimetry as a new method for biofilm investigations. This method measures heat released by metabolically active microorganisms. A potential bias due to the heat-insulating properties of the extracellular polymeric substances (EPS) on the measurement is assumed to be low since thermal conductivity of biofilms is in the same order as that of the bulk water (Characklis et al., 1981). The potential usage of the EPS as carbon source can be neglected if pure species biofilms are investigated and provided with enough carbon and energy. Therefore, heterogeneities of the biofilm are not expected to bias the result. Calorimetry has the advantages of being non-invasive and non-destructive. This opens avenues for long-term real-time monitoring of the growth and activity of biofilms. The application of calorimetry for biofilm investigation began in 1991, when cells were cultivated on glass beads, and the heat was measured in a microcalorimeter (Weppen et al., 1991). Despite this initial success, only few calorimetric biofilm studies have been reported thereafter (Wentzien et al., 1994; von Rege and Sand, 1998; Peitzsch et al., 2008; Hauser-Gerspach et al., 2008). This has been attributed to the limited flexibility and user-friendliness of commercially available calorimeters (Buchholz et al., 2010a).

For the present study, a recently developed chip-calorimeter was used. Its small size has the advantages of short thermal equilibration times, low medium consumption, and high flexibility. Its applicability to biological samples was tested for planktonic cells (Lerchner et al., 2011; Maskow et al., 2006) and biofilm cells (Maskow et al., 2006; Lerchner et al., 2008a). A previous spadework demonstrated the ability of chip-calorimetry to monitor the responses of biofilms to dosages of antibiotics and biocides (Buchholz et al., 2010b). However, questions regarding calorimetry-specific information had not been addressed due to technical shortcomings. Here, we compared chip-calorimetrically recorded killing curves with those obtained with common biofilm investigation methods (i.e. CFU counting, ATP measurements, and CLSM quantification). This served to further establish chip-calorimetry as a new method for biofilm research and to exploit the particular information content of calorimetric signals for a mechanistic understanding of biofilm killing.

This study was carried out using biofilms of *Pseudomonas putida* PaW340. This strain was used for bioremediation due to its ability to degrade aromatic hydrocarbons. Furthermore, this strain was found to be related to cases of infectious outbreaks (MacFarlane et al., 1991; Toru et al., 2008; Carpenter et al., 2008). In 2011, German TRBA (Technical Rules for Biological Agents) reclassified this strain into the higher risk group 2 (pathogens that can cause human or animal disease). Based on its lower number of reported infections, this strain can be judged less pathogenic than *P. aeruginosa*, and therefore was chosen for the present proof of principle study.

Materials and methods

Bacteria and biofilm cultivation

P. putida strain PaW340 (DSMZ 2112) was obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. For preservation, the strain was grown on Luria-Bertani (LB) agar plates overnight at $28 \pm 0.2\,^{\circ}C$ and afterwards stored at $4\,^{\circ}C$. Agar plates were re-inoculated every 4 weeks.

A colony was picked from the agar plate and transferred into a 100-ml Erlenmeyer flask containing 20 ml LB broth. The flask was put on a rotary shaker (150 rpm at $30 \pm 0.2\,^{\circ}C$) until the culture reached the stationary growth after approximately 24 h. The optical density was 3.0 ± 0.3 at 700 nm (measured with photometer Cary400Scan, Varian Deutschland GmbH, Darmstadt, Germany). The culture was used as the biofilm inoculum.

For the purpose of chip-calorimetric measurements, biofilms were cultivated in specific flow-through chambers (detailed in the section 'Chip-calorimeter' of 'Materials and methods'). Up to 12 chambers (for calorimetric analysis, measurements of CFU and ATP, or for quantitative CLSM) were prepared identically. The chambers were first chemically sterilized by flushing with a mixture of ethanol/water/sulphuric acid (1:0.41:0.01) for 20 min, followed by neutralizing with phosphate buffer for 1 h. The stationary *P. putida* culture was diluted in a defined medium composed of (in mg/l) NH_4Cl (764), $CaCl_2{\cdot}6H_2O$ (5.5), KH_2PO_4 (340), K_2HPO_4 (435), $MgCl_2{\cdot}6H_2O$ (59), $ZnCl_2$ (0.21), $MnCl_2$ (0.46), $CuCl_2$ (0.42), $Na_2MoO_4{\cdot}2H_2O$ (0.25), $FeCl_3{\cdot}6H_2O$ (4.0), sodium benzoate (600), and tryptophan (20) to an optical density of 0.2 (about 7×10^7 CFU/ml) and filled in 5-ml disposable syringes. Using an NE-1200 syringe pump (New Era Pump System, Inc., Farmingdale, USA), which holds up to 12 syringes, the cell suspension was continuously pumped through the chambers at a low flow rate of 3 ml/h. This phase for cell attachment was continued for 90 min. Subsequently, the syringes were replaced by 10-ml disposable syringes containing sterile, cell-free medium, and the biofilm was cultivated by pumping medium in a stop-flow mode through the chambers. The stop-flow mode consisted of an injection period during which 50 μl medium was pumped through the chamber at a rate of 6 ml/h (30 s) and a waiting period during which the medium was left in the chamber for 30 min. The stop-flow mode was chosen because it equals the alternating conditions during the subsequent measurements in the chip calorimeter.

After 24 h, one flow-through chamber was disconnected and mounted in the chip-calorimeter. The other chambers were used for reference measurements of CFU, ATP, or for quantitative CLSM. They were treated identically to the chamber mounted in the calorimeter regarding the flow and feeding regimes throughout the whole experiment. The cultivation on the flow-through chamber in the chip-calorimeter as well as in the reference experiments was continued for another 24 h before the established biofilms were treated with antibiotics.

Testing influences of antibiotics on biofilm

Two antibiotics representing different mechanisms of action were applied to biofilms, and their effects were monitored by various methods. The aminoglycoside kanamycin is bactericidal while tetracycline is bacteriostatic. These antibiotics were chosen because many biochemical details about their mode of action and the respective resistance mechanism of *Pseudomonas* strains are known. At time zero, the antibiotic concentration in the medium was increased from 0 to 30 or 60 mg/l. At distinct intervals, a chamber outside of the calorimeter was disconnected and used for CFU-, ATP-, or CLSM analysis.