Chip Calorimetry for Fast and Reliable Evaluation of Bactericidal and Bacteriostatic Treatments of Biofilms[▽]

F. Buchholz,¹ A. Wolf,² J. Lerchner,² F. Mertens,² H. Harms,¹ and T. Maskow^{1*}

UFZ-Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Leipzig, Germany,¹ and
TU Bergakademie Freiberg, Institute of Physical Chemistry, Freiberg, Germany²

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Chip calorimetry is introduced as a new monitoring tool that provides real-time information about the physiological state of biofilms. Its potential for use for the study of the effects of antibiotics and other biocides was tested. Established *Pseudomonas putida* biofilms were exposed to substances known to cause toxicity by different mechanisms and to provoke different responses of defense and resistance. The effects of these compounds on heat production rates were monitored and compared with the effects of these compounds on the numbers of CFU and intracellular ATP contents. The real-time monitoring potential of chip calorimetry was successfully demonstrated by using as examples the fast-acting poisons formaldehyde and 2,4-dinitrophenol (DNP). A dosage of antibiotics initially increased the heat production rate. This was discussed as being the effect of energy-dependent resistance mechanisms (e.g., export and/or transformation of the antibiotic). The subsequent reduction in the heat production rate was attributed to the loss of activity and the death of the biofilm bacteria. The shapes of the death curves were in agreement with the assumed variation in the levels of exposure of cells within the multilayer biofilms. The new monitoring tool provides fast, quantitative, and mechanistic insights into the acute and chronic effects of a compound on biofilm activity while requiring only minute quantities of the biocide.

The significance of the effects of biofilms on various medical problems is broadly accepted. Infectious kidney stones, bacterial endocarditis, and cystic fibrosis are related to the occurrence of biofilms. The formation of biofilms on implanted prosthetic devices may also cause serious problems. Multiresistant bacteria are isolated in increasing numbers in hospitals worldwide. The eradication of biofilm bacteria is therefore of major interest in present-day medicine, as can also be seen from the increasing number of related publications.

Biofilm cells represent the building blocks of complex multicellular structures and exhibit morphological and physiological differences from planktonic cells. One physiological peculiarity is the increased resistance of biofilm cells to antimicrobial agents, with the lethal doses for biofilm cells exceeding those for planktonic cells by factors of between 100 and 1,000 (13). Furthermore, biofilm bacteria are sheltered from many host defense mechanisms. Hence, antibiotic treatment and the monitoring of its effects need to be adapted to biofilms.

In many situations, the fast and, nevertheless, reliable determination of the bacterial reaction to antimicrobial agents is required, e.g., to test for biocidal activity or to prove successful disinfection. In view of the increasing rates of antibiotic resistance, the rising costs of therapeutic agents, and the demands of environmental protection, the sensible and well-directed application of antibiotics and biocides is advisable. Evaluations of efficacy tailored for biofilm cells should precede antibiotic treatment. Most available evaluation methods are limited by,

e.g., long processing times, incompatibility with high throughput, or the fact that the remaining biomass instead of activity or viability is determined (24). The staining of biofilms with, for instance, crystal violet or safranin, followed by washing, color extraction, and measurement of the absorption, is of low precision and addresses the biofilm mass rather than viability or activity (25). For viability testing, determination of the numbers of CFU is a very common technique. By that method, biofilms are removed by scraping, vortexing, or sonication; resuspended; and recultivated on agar plates to count the numbers of growing colonies (28). This technique is slow and error prone due to the possibility of incomplete resuspension before plating or the carryover of antibiotics. Direct biofilm visualization by confocal laser scanning microscopy or epifluorescence microscopy relies on the fluorescence of added 3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide, Syto, or Alexa dye. Besides being costly and toxic and requiring an environmentally friendly means of disposal, these dyes may interact with bacterial metabolism.

A promising alternative is to monitor biofilm activity by measurement of the metabolic heat. Calorimetric measurements are able to be obtained in a sensitive, rapid, noninvasive, and nondestructive manner. Calorimetry can easily be combined with other methods and provide precise real-time information. Calorimetry was introduced decades ago for use with suspended cells (16), and it was recently applied in medicine for the analysis of bacterial infections (2, 31, 32). Some new results reveal the applicability of calorimetry to biofilm research (10, 17). Calorimetry also appears to be a tool very well suited for investigation of the effects of chemicals on biofilms because it does not require disruption or recultivation of the biofilm. Growth and measurements can be carried out in the same vessel.

* Corresponding author. Mailing address: UFZ-Helmholtz Centre for Environmental Research, Department of Environmental Microbiology, Permoserstr. 15, Leipzig 04318, Germany. Phone: 49-341-235 1328. Fax: 49-341-235 1351. E-mail: Thomas.maskow@ufz.de.

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FIG. 1. Experimental setup for parallel biofilm cultivation (A) and the measurement principle of chip calorimetry (B).

Despite these clear advantages, calorimetry has only rarely been applied to biofilms and antibiotics (6, 18, 19). A microcalorimetric evaluation of the efficacies of biocides involving biofilm bacteria was successfully performed in 1998 (33). In that study, biofilms were grown on coupons of mild (unalloyed) steel, which were then transferred into the calorimeter cuvette. However, the suspended cells also contributed to the heat signal. This weakness can be overcome by using flowthrough systems, which, however, allow only low sample throughput. An alternative to conventional flowthrough calorimetry is the recently developed chip calorimetric technique. It is highly flexible and compatible with high throughput when exchangeable flowthrough cells are used (10). In the study described here, we tested the potential of chip calorimetry as a new tool for investigation of the effects of antibiotics on biofilm bacteria.

MATERIALS AND METHODS

Bacteria and culture conditions. To test the analytical potential of our recently developed chip calorimeter (10, 11), *Pseudomonas putida* strain PAW340 (DSMZ 2112) was used because it is one of the best investigated biofilm formers (20, 26). *Pseudomonads* are highly important in medicine as well as in biotechnology and are key degraders of organic pollutants in terrestrial ecosystems. The strain was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), maintained on agar slants with Luria-Bertani (LB) medium, and stored at 4°C. The cultures on agar plates were refreshed fortnightly. The liquid LB medium was used to prepare precultures for inoculation. Biofilms were established in the measurement chambers by connecting the chambers to a bioreactor and percolating the bacterial suspension through them. In the bioreactor, *P. putida* was grown in 100 ml of a defined medium composed of NH_4Cl (764 mg/liter), $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (5.5 mg/liter), KH_2PO_4 (340 mg/liter), K_2HPO_4 (435 mg/liter), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (59 mg/liter), ZnCl_2 (0.21 mg/liter), MnCl_2 (0.46 mg/liter), CuCl_2 (0.42 mg/liter), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.25 mg/liter), $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (4.0 mg/liter), and 500 mg/liter streptomycin. The defined medium was chosen to permit the calculation of the enthalpy balance. Streptomycin was added to obviate potential contamination because *P. putida* PAW340 possesses chromosomally encoded streptomycin resistance (up to 1 g/liter). As the carbon source, 600 mg/liter sodium benzoate was added. For longer cultivation periods, the substrate was not added at one time but was added in portions each day to prevent intoxication from high substrate concentrations. The bacterial culture was grown at 25°C, continuously stirred at 300 rpm, and aerated with compressed air. The pH was maintained constant at 7.0 ± 0.1 over the whole cultivation period due to the high buffering capacity of the medium. Occasional pH measurements indicated that this pH was stable throughout the experiment. The growing culture was pumped from the bioreactor through up to six flow chambers at a flow rate of 6 ml/h by use of a peristaltic pump (model 5058; Watson-Marlow GmbH, Rommelskirchen, Germany) equipped with Tygon tubings (inner diameter, 0.5 mm). Before the measurements were made, the planktonic cells were washed out with sterile medium. For sterilization, the entire flow system except the flow chamber was autoclaved at 121°C for 30 min. The sterilization of the flow chamber is described below.

Figure 1 shows schematically the setup used for the biofilm cultivation and a sketch of the calorimeter chip.

The calorimetric device. The calorimetric device was described in detail in a former publication (11). The main component is a silicon chip with integrated thin film thermopiles consisting of 118 BiSb/Sb thermocouples (1). The chip was produced by the Institute of Photonic Technology (IPHT), Jena, Germany. It converts the heat generated by the bacteria inside the measurement chamber into a voltage signal. The average sensitivity is 8.3 V/W, as determined by a chemical calibration which was established for conventional microcalorimeters (23). The measurement chamber is made from polymethylmethacrylate (PMMA). The flow channel is 20 mm long, 1.0 mm wide, and 0.5 mm high; and the volume is 10 μl . After cultivation of the biofilm, the measurement chamber is mounted on the chip. The complete chip module is enclosed in a high-precision thermostat with a temperature control capability better than 10 μK . Before the addition, the substrate solution is thermally equilibrated in heat exchangers close to the chip module. The top and the bottom of the flow channel are covered by PMMA foils (Goodfellow, Bad Nauheim, Germany) 50 μm thick. The foils can be replaced to vary the substratum for biofilm deposition. The detection limit of the calorimeter was 20 nW, which corresponds to an early-stage biofilm of approximately 3×10^5 bacteria/ cm^2 (10). The measurement temperature corresponds to the biofilm cultivation temperature of 25°C.

Experimental procedures. Prior to the calorimetric measurements, the fluidic system of the calorimeter was chemically disinfected with a mixture of ethanol-sulfuric acid (1:0.41:0.01) for 30 min, followed by cleaning with autoclaved distilled water and a final flush with substrate solution. After mounting of the measurement chamber containing the biofilm to the calorimetric system, 20 to 30 min was allowed for thermal equilibration.

Calorimetric signals were generated by injecting nutrient solution into the biofilm-colonized measurement chamber. A stopped-flow measurement regimen with alternating injection and waiting periods was applied in order to correct for baseline drifts. After an injection, the signal returns to the baseline due to oxygen depletion. This signal was taken as a reference value for measurement of the heat production rate. The small thermal time constant of the calorimeter of about 12 s enables excellent real-time measurements to be used for the drift corrections, as already described by Lerchner et al. (10). The chambers used for comparison of the calorimetric method with the conventional methods of analysis of biofilms (e.g., CFU determination and measurement of the intracellular ATP content) were treated by use of the same stopped-flow mode applied for the calorimetric monitoring. For such reference measurements, chambers were removed from the flow line at distinct times and analyzed.

Investigation of antibiotic effects. The response of a *P. putida* biofilm to selected antimicrobial chemicals was investigated. The selection was based on different toxicity targets and different bacterial defense and resistance mechanisms. The chemicals are known to affect the replication machinery, protein synthesis, and energy generation or to act nonspecifically. Table 1 summarizes the chemicals applied and describes the respective toxicity targets and a few main mechanisms of defense and resistance. Bacteriostatic and bactericidal substances were included. The resistance mechanisms comprise export systems and transformation and modification of the target structures. Tetracycline and 2,4-dinitrophenol (DNP) were obtained from Merck KGaA, Darmstadt, Germany; kanamycin was obtained from Carl Roth GmbH + Co. KG, Karlsruhe, Germany; and ciprofloxacin was obtained from AppliChem GmbH, Darmstadt, Germany. All chemicals were of analytical purity grade. The susceptibility of *P. putida* to these substances was tested in agar diffusion tests. For the study of antibacterial effects,

TABLE 1. Chemicals used to test chip calorimetry for detection of the effects of antimicrobials on biofilms

Substance/type of toxicant	Toxicity mechanism	Main resistance mechanism (29, 34)
Tetracycline/antibiotic	Bacteriostatic; at low concentrations, inhibition of translation via reversible blocking of the amino-acyl tRNA binding site at the ribosome; at high concentrations, further toxic effects such as inhibition of the respiratory chain, interference with DNA synthesis, and complexation of essential trace elements	Export of the antibiotic (36)
Kanamycin/antibiotic	Bactericidal; irreversible binding to ribosome and 16S rRNA, leading to the misreading of mRNA, and insertion of wrong amino acids, yielding nonfunctional or toxic peptides; interaction with EPS (36)	Enzymatic modification of the drug (34)
Ciprofloxacin/antibiotic	Bactericidal; inhibition of topoisomerases II and IV, which are essential for DNA replication, transcription, repair, and recombination; no interaction with EPS reported	Modification of target structure via mutation of DNA gyrase genes (34)
DNP/uncoupling agent	Uncoupler; uncoupling of ATP formation from respiratory electron transport	Enhanced respiration; export of the biocide (28)
Formaldehyde/nonspecific poison	Bactericidal; nonspecific reactions with proteins and nucleic acids	Transformation of the biocide via formaldehyde dehydrogenase (5, 12, 29)

the substances were added to the nutrient solution at different concentrations, and the calorimetric measurements were obtained as described above.

For determination of the numbers of CFU, a flow cell was disconnected from the chip calorimeter or nutrient solution loop and flushed with sterile air to remove the suspended cells. The chamber was filled with carbon substrate-free medium. The inlets and outlets were closed with sterilized tubes. To release the cells from the biofilm structure, the chambers were treated three times in an ultrasonic bath and were vortexed afterwards (8). Finally, the flow cell was flushed with 90 μ l medium, and the contents of the chamber and the flushing solution were pooled. The numbers of CFU were determined by plating serially diluted 100- μ l aliquots on LB agar slants. This was done before the antibiotic treatment and after the completion of the experiment.

ATP measurements. As a reference for the calorimetric measurements, a few biofilm samples were analyzed for intracellular ATP concentrations. ATP was extracted from the biofilms in the reference flowthrough chamber by flushing the chambers three times at 2-min intervals with 50 μ l of 1.3 M perchloric acid containing 23 mM EDTA with a microsyringe. The samples were neutralized with the required amount of 0.72 M KOH amended with 0.16 M KHCO₃. The precipitates were separated by centrifugation at $8,064 \times g$ for 10 min. The supernatants were stored at -20°C until further analysis.

Determination of the ATP contents was performed with an ATP kit SL (Biotherma, Handen, Sweden) which contained the required ATP monitoring solution, buffer, and an ATP standard. The measurement principle that the kit uses relies on the luciferase reaction. An internal standard of 10 μ M ATP was applied for quantification. The samples with internal standard were measured in duplicate; those without were measured in triplicate. A 96-well black-and-white microtiter plate was used. The addition of the chemicals was carried out automatically right before measurement with a spectrofluorometric device (Wallac Victor 1420 multilabel counter; Perkin Elmer-LAS GmbH, Rodgau-Jügesheim, Germany).

RESULTS

The applicability of calorimetry as an analytical tool for the actions of antibiotics/biocides on biofilms was tested by using as examples several chemicals acting on different target structures in biofilm organisms and provoking different physiological responses (Table 1).

Tetracycline. Tetracycline was included as an example of a bacteriostatic agent. Figure 2 shows the effects of the continuous treatment of *P. putida* biofilms with tetracycline at different dosages. Prior to the addition of the antibiotic, the biofilms were cultivated until a stable signal was obtained. For easier comparison, these reference signals were set equal to 100%. When tetracycline was added, the calorimetric signals responded immediately and in a dosage-dependent manner. Two

FIG. 2. (A) Response of the rate of heat production of a *P. putida* biofilm to exposure to different, continuously supplied concentrations of tetracycline (TET). The signal intensities are normalized to the last values before the addition of the antibiotic. The large symbols indicate the phase of the rate of increased heat production. (B) The maximum increment in the rate of heat production rate directly after exposure to different tetracycline concentrations (phase 1).

FIG. 3. Rate of heat production of a *P. putida* biofilm continuously treated with different amounts of kanamycin (KAN). The signal intensities are normalized to the last values before the addition of the antibiotic.

phases of the response, i.e., an immediate rise and then a slow decline, were distinguished.

The increment in the heat production rate (Fig. 2B) had a maximum of 118% at 70 µg/ml and approached the reference

level (100%) at very low (3 µg/ml) and very high (250 µg/ml) tetracycline concentrations.

The concentration dependence of the inactivation rate (IR) in the second phase can be described by a constant value (standard deviation = 0.30% signal decrease per h) below the threshold of 70 µg ml⁻¹ and by a quadratic function ($IR = 9.63 \cdot 10^{-5} \text{ liter}^2 \mu\text{g}^{-2} \% \text{ h}^{-1} \text{ TET}^2 - 4.87 \cdot 10^{-4} \text{ liter } \mu\text{g}^{-1} \% \text{ h}^{-1} \text{ TET} + 0.30\% \text{ h}^{-1}$, where TET is the tetracycline concentration) above the threshold.

Kanamycin. Kanamycin was tested as an agent known to exert bactericidal activity and to interact with extracellular polymeric substances (EPSs). The quick increase in the rate of heat production immediately after the antibiotic was applied and then by a slow decline was similar to what was observed with tetracycline (Fig. 3). However, kanamycin was more effective, and the inactivation of the *P. putida* biofilm in the second phase was independent of the kanamycin concentration. The decline became faster with longer exposure times.

Ciprofloxacin. Ciprofloxacin was included as a bactericidal agent that does not interact with EPSs. The response of the *P. putida* biofilms to ciprofloxacin (Fig. 4, top panel) was more complex than the responses to tetracycline and kanamycin. At low concentrations, a slight increase in the rate of heat production was seen. The logarithmic drop in the rate of heat

FIG. 4. Response of a *P. putida* biofilm continuously treated with different doses of ciprofloxacin (CIP). (Top) Rate of heat production; (bottom) comparison of heat and ATP levels. The signal intensities are normalized to the last values before the addition of the antibiotic.

FIG. 5. (A) Response of the heat production of a *P. putida* biofilm to two pulses of DNP. (B) Rate of heat production and ATP content of a *P. putida* biofilm continuously treated with 0.5 mM DNP. The signal intensities are normalized to the last values before the addition of the uncoupler.

production during the second phase was concentration dependent and became faster with time.

In the experiments with ciprofloxacin, further indicators of activity (i.e., the intracellular ATP content to describe anabolism and the rate of heat production mainly to describe catabolism) were analyzed and represent the different killing kinetics (Fig. 4, bottom).

DNP. DNP was chosen as an agent that attacks energy generation by uncoupling electron transport phosphorylation and ATP synthesis. Figure 5A shows that *P. putida* responded immediately to the addition of DNP, with the heat signal declining drastically. A switch back to DNP-free nutrient solution led to a gradual, nearly complete return to the initial heat signal. A second addition of DNP-containing nutrient solution led to a similar decline, followed by a nearly complete return upon the removal of the DNP.

The changes in the metabolic heat production rate upon the continuous addition of a lower DNP concentration (0.5 mM) were correlated to the concentrations of extractable ATP. The continuously decreasing heat production rate went along with

FIG. 6. Rate of heat production of a *P. putida* biofilm treated with 2% formaldehyde. The signal intensities are normalized to the last values before the addition of the biocide.

an initially declining ATP content, which after some time returned to the original value.

Formaldehyde. Formaldehyde was selected as an agent that reacts nonspecifically with amino acids and proteins, thereby damaging vital cellular structures. The metabolic activity of the biofilm dropped immediately after the addition of a 2% formaldehyde solution (Fig. 6). Replacement with a formaldehyde-free nutrient solution did not lead to the recovery of the metabolic activity (results not shown).

DISCUSSION

We tested the analytical potential of chip calorimetry in some detail by monitoring the changes in the rate of heat production of *P. putida* biofilms upon treatment with antibiotics acting on different cellular targets, an uncoupling agent, and a nonspecific toxicant. The rates of heat production recorded were compared with the results of conventional methods of biofilm analysis, namely, determination of the numbers of CFU and measurement of the intracellular ATP contents. In the following discussion, we will try to interpret the peculiarities of the reactions to our test chemicals.

Tetracycline is known to act bacteriostatically by inhibiting the early steps of protein biosynthesis (37) and by interfering with the respiratory chain and DNA synthesis at higher concentrations. Most of the known mechanisms of resistance to tetracycline, e.g., the removal of the antibiotic by high-affinity efflux pumps, require biologically usable energy (ATP, GTP, acetyl groups, etc.) (36). This energy demand needs to be satisfied by enhanced catabolic activity (e.g., electron transport phosphorylation) and can explain the observed enhanced rate of heat production after dosing with tetracycline. The observation that maximum heat production is followed by a decrease can be attributed to the deactivation of some biofilm cells. After a while and particularly in the presence of higher tetracycline concentrations, parts of the biofilm cannot withstand the bacteriostatic activity of tetracycline. This deactivation was confirmed by reductions in the numbers of CFU (results not shown). Besides cellular defense mechanisms, protective ef-

fects also arise from the biofilm architecture and its biological heterogeneity. The mechanisms of protection specific to biofilms may include (i) the slow penetration of the antimicrobial agent, leading to different levels of exposure for individual cells; (ii) the physiological heterogeneity of the biofilm population; and (iii) the presence of phenotypic variants or persister cells (3, 5, 14). The failure of purely cellular resistance would be reflected by logarithmic inactivation kinetics, whereas the superimposed effects of biofilm-specific resistance mechanisms would bring about more complex kinetics. For instance, the slow penetration of the toxicant would lead to a continuum of exposure regimens as cells closer to the biofilm basis would experience the antibiotic later and a slower buildup of the applied concentration. As more and more cells become exposed to the antibiotic, one would expect an acceleration of the inactivation rate with time. The presence of persister cells would lead to biphasic inactivation kinetics, as those cells would resist and outcompete more susceptible cells. The linear shape of the inactivation curve (log [heat production rate] versus time) for tetracycline formally fits models of the deployment of adaptive stress responses, i.e., the transformation of susceptible cells to adapted cells which are more stable to the antimicrobial agent. Details of the respective simulations and the necessary assumptions are given elsewhere (4).

The lowest tetracycline concentration influencing the heat signal (3 $\mu\text{g/ml}$) within 10 h was far below the concentrations typically required to eradicate a biofilm. It was in the range of MICs reported for planktonic cells of potentially pathogenic bacterial strains (*Escherichia coli*, 0.07 to 2.5 $\mu\text{g/ml}$; *Klebsiella pneumoniae*, 2.5 to 10 $\mu\text{g/ml}$; *Staphylococcus aureus*, 0.15 $\mu\text{g/ml}$; *Pseudomonas aeruginosa*, 6.25 to 12.5 $\mu\text{g/ml}$) (27).

Kanamycin acts as a bactericide, meaning that it actively kills bacteria. This is reflected by the observed decrease in the heat signal, which was faster with kanamycin than with tetracycline at the same dosage. It is also reported that aminoglycosidic antibiotics such as kanamycin interact with EPSs (35). As long as free binding sites for kanamycin are available, the level of penetration of the free antibiotic is reduced and the inactivation is expected to have a low concentration dependence. This would explain why the observed killing curve has little concentration dependence. Indeed, our observation matches the typical bilinear pattern simulated by Chambless and coworkers (4) for the model of slow penetration. Bacteria typically counteract aminoglycosides such as kanamycin by enzymatic modification of the antibiotics (e.g., N-acetylation, O-phosphorylation, or O-adenylylation) (34). These modifications require biologically usable energy and thus initially enhance the heat production rate, as was observed. Kanamycin concentrations of 3 to 30 $\mu\text{g/ml}$ showed clear effects on the rate of heat production rate and are in the range of MICs that have been described (2 to 32 $\mu\text{g/ml}$ for *Neisseria gonorrhoeae* [12]).

In the case of ciprofloxacin, no energy-requiring resistance mechanisms are described. Walsh discussed a DNA gyrase mutation as being a drug resistance mechanism and referred to it as a reprogramming of the target structure (34). If a mutation probability of 1 to 10^7 (34) and doubling times of 120 min (26) are assumed, reprogramming is much too slow to be detected in our experiments. Furthermore, such a resistance mechanism will have a low demand for biologically usable energy and will thus not influence the calorimetric signal.

Staphylococcus strains are known to resist fluoroquinolone antibiotics such as ciprofloxacin using the chromosomally encoded ATP-driven efflux pump NorA (21). The slight enhancement in the rate of heat production that we observed after antibiotic dosing may indicate the active export of ciprofloxacin in *P. putida* as well. The observed bilinear shape of the inactivation curves matches the model of slow antibiotic penetration. The numbers of CFU were strongly reduced over the concentration range investigated (20 to 150 $\mu\text{g/ml}$), supporting our calorimetric measurements.

The hydrophobic, weak acid DNP is protonophoric (29). It shuttles protons into the cell, thereby diminishing the proton gradient. The electron transport in the respiratory chain is thus uncoupled from the generation of ATP. DNP thus leads to the accelerated consumption of the carbon substrate to compensate for the loss of biologically usable energy. The expected consequence is enhanced heat production. In contrast to this expectation, the addition of DNP to the biofilm decreased the heat signal by roughly 90% within 1 h. One reason could be that benzoate degradation is initiated by the NAD(P)H-dependent toluate-1,2-dioxygenase (<http://biocyc.org>). It appears likely that uncoupling depletes the NAD(P)H pool, thereby interfering with the initial attack of benzoate. Furthermore, energy uncoupling by DNP could reduce the level of active benzoate uptake, with reduced heat production being a consequence. An early investigation of benzoate transporters (30) and a recent analysis of the gene products of *P. putida* benzoate transporters (22) support the active uptake of benzoate by *P. putida*. The observation of reduced ATP contents and the immediate recovery of heat production upon the removal of DNP support our hypothesis that the interference with energy-dependent uptake or catabolic reactions rather than irreversible intoxication reduced the rate of heat production. Continuous treatment of *P. putida* with DNP resulted in permanently reduced heat production, whereas the ATP content recovered after some time. We hypothesize that sublethal concentrations of DNP allow cellular adaptations, such as changes in membrane permeability, to take place as a result of variation of the lipid fatty acid composition, the actions of extrusion pumps, low-energy-shock adaptive responses, or a switch to Na^+ -based energetics. All these mechanisms have been discussed as bacterial resistance mechanisms against protonophores (9, 15). They would allow ATP synthesis to be maintained to a certain extent.

The results of our experiments with the nonspecific poison formaldehyde impressively demonstrate the real-time monitoring properties of chip calorimetry. Although bacteria may counteract formaldehyde poisoning by oxidation of the toxicant, which even generates a reduction in power, i.e., NAD(P)H, high concentrations such as those applied in the present study damage vital cell structures too quickly for such a defense to become active and calorimetrically visible.

Figure 7 summarizes the rate of heat production rate of *P. putida* biofilms subjected to antibiotics/biocides that have different mechanisms of action (the bacteriostatic agent tetracycline, the bactericidal agent kanamycin, the uncoupling agent DNP, and the nonspecifically acting agent formaldehyde). The different modes of action can be clearly distinguished. As an example of a nonspecific poison, formaldehyde immediately shuts down any metabolic activity. Kanamycin as an example of

FIG. 7. Calorimetrically monitored responses of a *P. putida* biofilm to the bacteriostatic agent tetracycline, the bactericidal agent kanamycin, the uncoupling agent DNP, and the nonspecific toxicant formaldehyde.

a bactericidal antibiotic that more rapidly reduces the rate of heat production rate than a bacteriostatic antibiotic. Such information cannot be generated by the usual CFU approach.

Conclusions. Our results show that chip calorimetry can indicate physiological changes in biofilms in real time and provide some information about pharmacokinetics and resistance mechanisms. The use of calorimetric measurements is not restricted to aerobic biofilms. Microorganisms that use other terminal electron acceptors (e.g., Fe^{3+} , NO_3^- , SO_4^{2-} , and organic intermediates) as well as biofilms that are likely to form redox gradients can be advantageously investigated calorimetrically. While chip calorimetry is a self-standing method for the monitoring of disinfection capabilities, the use of calorimetry in combination with other analytical tools may provide additional in-depth information on the biochemistry behind the action of an antibiotic. This could largely go beyond the use of the demonstrated combination of calorimetry with CFU and ATP measurements. The use of calorimetry in combination with live-dead staining, cytometric proliferation activity analysis, transcriptomics, functional enzyme activities, etc., could be imagined.

We were able to perform an economical and fast investigation of the effects of biofilm treatment. This was achieved by separating biofilm cultivation in exchangeable chambers from the calorimetric measurements and employing calorimeter chips with a low heat capacity and correspondingly shorter thermal equilibration times as conventional calorimeters.

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REFERENCES

- Baier, V., R. Foedisch, A. Ihring, E. Kessler, J. Lerchner, and G. Wolf. 2005. Highly sensitive thermopile heat power sensor for micro-fluid calorimetry of biochemical processes. *Sens. Actuators A* 123–124:354–359.
- Baldoni, D., H. Hermann, R. Frei, A. Trampuz, and A. Steinhuber. 2009. Performance of microcalorimetry for early detection of methicillin resistance in clinical isolates of *Staphylococcus aureus*. *J. Clin. Microbiol.* 47:774–776.
- Brown, M. R. W., D. G. Allison, and P. Gilbert. 1988. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J. Antimicrob. Chemother.* 22:777–780.
- Chambless, J. D., S. M. Hunt, and P. S. Stewart. 2006. A three-dimensional computer model of four hypothetical mechanisms protecting biofilms from antimicrobials. *Appl. Environ. Microbiol.* 72:2005–2013.
- Davies, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nat. Rev. Drug. Discov.* 2:114–122.
- Gaisford, S., A. E. Beezer, A. H. Bishop, M. Walker, and D. Parsons. 2009. An in vitro method for the quantitative determination of the antimicrobial efficacy of silver-containing wound dressings. *Int. J. Pharm.* 366:111–116.
- Reference deleted.
- Hamilton, B., J. Heersink, K. Buckingham-Meyer, and M. Goeres. 2003. The biofilm laboratory. Center for Biofilm Engineering, Montana State University, Bozeman, MT.
- Krulwich, T. A., P. G. Quirk, and A. A. Guffanti. 1990. Uncoupler-resistant mutants of bacteria. *Microbiol. Mol. Biol. Rev.* 54:52–65.
- Lerchner, J., A. Wolf, F. Buchholz, F. Mertens, T. R. Neu, H. Harms, and T. Maskow. 2008. Miniaturized calorimetry—a new method for real-time biofilm activity analysis. *J. Microbiol. Methods* 74:74–81.
- Lerchner, J., A. Wolf, H. J. Schneider, F. Mertens, E. Kessler, V. Baier, A. Funfak, M. Nietzsch, and M. Krügel. 2008. Nano-calorimetry of small-sized biological samples. *Thermochim. Acta* 477:48–53.
- Lesmana, M., C. I. Lebron, D. Taslim, P. Tjaniadi, D. Subekti, M. O. Wasfy, J. R. Campbell, and B. A. Oyoyo. 2001. In vitro antibiotic susceptibility of *Neisseria gonorrhoeae* in Jakarta, Indonesia. *Antimicrob. Agents Chemother.* 45:359–362.
- Lewis, K. 2005. Persister cells and the riddle of biofilm survival. *Biochemistry (Mosc.)* 70:267–274.
- Lewis, K. 2007. Persister cells, dormancy and infectious disease. *Nat. Rev. Microbiol.* 5:48–56.
- Lewis, K., V. Naroditskaya, A. Ferrante, and I. Fokina. 1994. Bacterial resistance to uncouplers. *J. Bioenerg. Biomembr.* 26:639–646.
- Maskow, T., R. Kemp, F. Buchholz, T. Schubert, B. Kiesel, and H. Harms. 1 June 2009, posting date. What heat is telling us about microbial conversions in nature and technology: from chip- to megacalorimetry. *Microb. Biotech.* doi: 10.1111/j. 1751-7915.2009.00121.x.
- Maskow, T., J. Lerchner, M. Peitzsch, H. Harms, and G. Wolf. 2006. Chip calorimetry for the monitoring of whole cell biotransformation. *J. Biotechnol.* 122:431–442.
- Morgan, T. D., and A. E. Beezer. 1998. An acrylic ampoule represents a denture surface in a microcalorimetric biofilm problem. *Microbios* 95:55–64.
- Morgan, T. D., A. E. Beezer, J. C. Mitchell, and A. W. Bunch. 2001. A microcalorimetric comparison of the anti-*Streptococcus mutans* efficacy of plant extracts and antimicrobial agents in oral hygiene formulations. *J. Appl. Microbiol.* 90:53–58.
- Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. P. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, and M. Holmes. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* 4:799–808.
- Neyfakh, A. A., C. M. Borsch, and G. W. Kaatz. 1993. Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrob. Agents Chemother.* 37:128–129.
- Nishikawa, Y., Y. Yasumi, S. Noguchi, H. Sakamoto, and J. Nikawa. 2008. Functional analyses of *Pseudomonas putida* benzoate transporters expressed in the yeast *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.* 72: 2034–2038.
- O'Neill, M. A., A. E. Beezer, C. Labetoulle, L. Nicolaidis, J. C. Mitchell, and J. A. Orchard. 2003. The base catalysed hydrolysis of methyl paraben: a test reaction for flow microcalorimeters used for determination of both kinetic and thermodynamic parameters. *Thermochim. Acta* 399:63–71.
- Pettit, R. K., C. A. Weber, M. J. Kean, H. Hoffmann, G. R. Pettit, R. Tan, K. S. Franks, and M. L. Horton. 2005. Microplate Alamar blue assay for *Staphylococcus epidermidis* biofilm susceptibility testing. *Antimicrob. Agents Chemother.* 49:2612–2617.
- Polonio, R. E., L. A. Mermel, G. E. Paquette, and J. F. Sperry. 2001. Eradication of biofilm-forming *Staphylococcus epidermidis* (RP62A) by a combination of sodium salicylate and vancomycin. *Antimicrob. Agents Chemother.* 45:3262–3266.
- Sauer, K., and A. K. Camper. 2001. Characterization of phenotypic changes in *Pseudomonas putida* in response to surface-associated growth. *J. Bacteriol.* 183:6579–6589.
- Shah, P. M., and W. Stille. 1973. Über die antibakterielle Wirkung subinhibitorischer Tetracyclin-Konzentrationen. *Infection* 1:110–112.
- Spoering, A. L., and K. Lewis. 2001. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J. Bacteriol.* 183:6746–6751.
- Terada, H. 1990. Uncouplers of oxidative phosphorylation. *Environ. Health Perspect.* 87:213–218.
- Thayer, J. R., and M. L. Wheelis. 1982. Active transport of benzoate in *Pseudomonas putida*. *J. Gen. Microbiol.* 128:1749–1753.

31. **Trampuz, A., S. Salzmann, J. Antheaume, and A. U. Daniels.** 2007. Microcalorimetry: a novel method for detection of microbial contamination in platelet products. *Transfusion* **47**:1643–1650.
32. **Trampuz, A., A. Steinhuber, M. Wittwer, S. L. Leib, R. J. Derksen, V. M. H. Coup, M. W. van Tulder, B. Veenings, F. C. Bakker, and A. Conen.** 2007. Rapid diagnosis of experimental meningitis by bacterial heat production in cerebrospinal fluid. *BMC Infect. Dis.* **7**:116–122.
33. **von Rege, H., and W. Sand.** 1998. Evaluation of biocide efficacy by microcalorimetric determination of microbial activity in biofilms. *J. Microbiol. Methods* **33**:227–235.
34. **Walsh, C.** 2000. Molecular mechanisms that confer antibacterial drug resistance. *Nature* **406**:775–781.
35. **Wright, G. D.** 2007. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* **5**:175–186.
36. **Zakeri, B., and G. D. Wright.** 2008. Chemical biology of tetracycline antibiotics. *Biochem. Cell Biol.* **86**:124.