Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*

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Received 27 November 2004

Revised 26 May 2005

Accepted 22 July 2005

Bacterial cultures produce subpopulations of cells termed 'persisters', reputedly known for high tolerance to killing by antibiotics. Ecologically, antibiotics produced by competing microflora are only one potential stress encountered by bacteria. Another pressure in the environment is toxic metals that are distributed ubiquitously by human pollution, volcanic activity and the weathering of minerals. This study evaluated the time- and concentration-dependent killing of *Escherichia coli* planktonic and biofilm cultures by the water-soluble metal(loid) oxyanions chromate (CrO_4^{2-}), arsenate (AsO_4^{3-}), arsenite (AsO_2^-), selenite (SeO_3^{2-}), tellurate (TeO_4^{2-}) and tellurite (TeO_3^{2-}). Correlative to previous reports in the literature, control antibiotic assays indicated that a small proportion of *E. coli* biofilm populations remained recalcitrant to killing by antibiotics (even with 24 h exposure). In contrast, metal oxyanions presented a slow, bactericidal action that eradicated biofilms. When exposed for 2 h, biofilms were up to 310 times more tolerant to killing by metal oxyanions than corresponding planktonic cultures. However, by 24 h, planktonic cells and biofilms were eradicated at approximately the same concentration in all instances. Coloured complexes of metals and chelators could not be generated in biofilms exposed to CrO_4^{2-} or TeO_3^{2-} , suggesting that the extracellular polymeric matrix of *E. coli* may have a low binding affinity for metal oxyanions. Viable cell counts at 2 and 24 h exposure revealed that, at high concentrations, all of the metal oxyanions had killed 99 % (or a greater proportion) of the bacterial cells in biofilm populations. It is suggested here that the short-term survival of < 1 % of the bacterial population corresponds well with the hypothesis that a small population of persister cells may be responsible for the time-dependent tolerance of *E. coli* biofilms to high concentrations of metal oxyanions.

INTRODUCTION

Biofilms are surface-adherent microbial consortia encased in a matrix of extracellular polymeric substance (EPS). The natural development of these microbial communities is regarded as part of the normal life cycle of the vast majority of bacteria, both in nature and in disease (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004). Biofilms play a pivotal role in the biochemical cycling of minerals in the environment (Brown *et al.*, 1999) and have an infamous

ability to survive high concentrations of antimicrobials, including metals. As an example, biofilms of the soil bacterium *Pseudomonas aeruginosa* have been reported to be two to 600 times more tolerant to the heavy metals Cu^{2+} , Zn^{2+} and Pb^{2+} than planktonic cells (Teitzel & Parsek, 2003; Harrison *et al.*, 2005a). Biofilms are also known for their ability to trap charged compounds in their EPS matrix (Stewart, 2003; Harrison *et al.*, 2005a). This has served as a novel approach to detect industrial emissions in rivers (Mages *et al.*, 2004). Very few studies have focused on biofilm tolerance to heavy-metal and -metalloid oxyanions. These compounds have a significant ecological impact and are spread into the environment through industry, volcanism and the natural erosion of minerals (reviewed by Brown *et al.*, 1999). In particular, oxyanions of chromium, arsenic, selenium and tellurium are prevalent pollutants with high biological toxicity (reviewed by Cervantes *et al.*, 2001; Mukhopadhyay *et al.*, 2002; Chasteen & Bentley, 2003; Oremland & Stolz, 2003).

Abbreviations: CLSI, Clinical Laboratory Standards Institute; DAPA, diaminopimelic acid; EPS, extracellular polymeric substance; GSH, reduced glutathione; HTP, high-throughput; MBC, minimum bactericidal concentration; MBEC, minimum biofilm-eradication concentration; Na_2DDTC , sodium diethyldithiocarbamate; SEM, scanning electron microscopy.

Supplementary figures are available with the online version of this paper.

Biofilm tolerance to antibiotics and disinfectants is dependent on many factors (reviewed by Lewis, 2001). Metal tolerance in bacterial biofilms is similarly multifactorial (Harrison *et al.*, 2005b). Factors such as metabolic heterogeneity, due to micronutrient and oxygen restriction (Huang *et al.*, 1998; Xu *et al.*, 2000; Stewart, 2002; Walters *et al.*, 2003), or changes in physiology due to quorum sensing-regulated gene expression (Beloin *et al.*, 2004; Stanley & Lazazzera, 2004) may play a role. Restricted diffusion and/or penetration of charged molecules into the biofilm matrix may also be a contributing factor (Stewart *et al.*, 2001; Stewart, 2003). It has also been hypothesized that growth stage-dependent production of specialized survivor cells, termed 'persisters', may mediate the biofilm tolerance phenomenon (Spoering & Lewis, 2001; Keren *et al.*, 2004b; Harrison *et al.*, 2005a). This latter hypothesis is the focus of the present study.

All genetically homogeneous bacterial populations (tested to date) produce a subpopulation of cells that survive prolonged exposure to high concentrations of bactericidal antibiotics (Bigger, 1944; Moyed & Bertrand, 1983; Lewis, 2001; Spoering & Lewis, 2001; Balaban *et al.*, 2004; Keren *et al.*, 2004a; Harrison *et al.*, 2005a). These 'persister' cells typically occur at a frequency of 10^{-6} to 10^{-5} in planktonic populations of *Escherichia coli* (Moyed & Bertrand, 1983). It has been hypothesized that this proportion may be as high as 10^{-2} in biofilms (Spoering & Lewis, 2001). A hallmark of the persister population is biphasic killing kinetics by antibiotics that are (i) time-dependent (Balaban *et al.*, 2004; Keren *et al.*, 2004a) and/or (ii) concentration-dependent (Brooun *et al.*, 2000; Spoering & Lewis, 2001). Persisters have been associated with a slow-growth phenotype that was identified by using specialized microfluidic devices and fluorescent microscopy (Balaban *et al.*, 2004). Strictly speaking, the persistent phenotype is not considered 'resistant' because these cells do not grow at high concentrations of antimicrobials. Rather, persisters simply do not die and thus exhibit multidrug tolerance (Keren *et al.*, 2004b).

The frequency at which persisters occur in populations of *E. coli* is regulated by the expression of chromosomal toxin-antitoxin genes (Keren *et al.*, 2004b). The best-studied example is the toxin-antitoxin pair *hipAB*. When these genes are deleted from the chromosome, stationary-phase planktonic cells and biofilms of *E. coli* have decreased tolerance to ciprofloxacin and mitomycin C (Keren *et al.*, 2004b). Overexpression of *hipA* is also correlated to joint tolerance of clinically isolated *E. coli* to β -lactam and fluoroquinolone antibiotics (Falla & Chopra, 1998). Kim Lewis and colleagues have hypothesized that expression of toxins drives bacteria reversibly into the slow-growing, multidrug-tolerant phenotype by 'shutting down' targets of antibiotics (reviewed by Lewis, 2005). Expression of the cognate antitoxins causes bacteria to exit this physiological state (Keren *et al.*, 2004b). Persisters thus represent a recalcitrant nidus that can seed a new population with

normal susceptibility once the antibiotic is removed (Bigger, 1944; Moyed & Bertrand, 1983; Spoering & Lewis, 2001; Harrison *et al.*, 2004a; Keren *et al.*, 2004a).

Unlike antibiotics, which generally have a single, distinct and highly specific target, metal and metalloid oxyanions may exert toxicity through many mechanisms. Metals may have high-affinity and energetically favourable, site-specific reactions with a target biomolecule. An example is the Painter-type reaction of tellurite (TeO_3^{2-} with glutathione, a reaction that has been observed to occur rapidly *in vivo* with studies using *E. coli* (Turner *et al.*, 2001). Displacement of metals with similar reactivity from proteins may alter biological activity of the target molecule (Schützendübel & Polle, 2002). Preliminary evidence also suggests that some heavy-metalloid oxyanions (in particular, TeO_3^{2-}) may exert toxicity by destroying the proton-motive force of cell membranes (Lohmeier-Vogel *et al.*, 2004). Metals may produce hydroxyl and superoxide radicals via reduction or Fenton-type reactions (Stohs & Bagchi, 1995; Inaoka *et al.*, 1999; Geslin *et al.*, 2001). Finally, metals may also exert toxicity through oxidative stress exerted on the free thiol groups of proteins (Stohs & Bagchi, 1995; Turner *et al.*, 1998, 1999).

In this study, we investigated whether bacterial persistence may play a role in the tolerance of *E. coli* JM109 to the toxicity of metal oxyanions. We chose six water-soluble heavy-metal and -metalloid oxyanions: chromate (CrO_4^{2-}), arsenate (AsO_4^{3-}), arsenite (AsO_2^-), selenite (SeO_3^{2-}), tellurate (TeO_4^{2-}) and tellurite (TeO_3^{2-}). With 2 h exposure, we report that biofilms were 1.8–310 times more tolerant to metal oxyanions than corresponding planktonic cultures. However, with 24 h exposure, metal oxyanions eradicated biofilm and planktonic cultures at approximately the same concentration in every instance. Subsequently, we evaluated biofilm and planktonic viable cell counts for a range of metal-oxyanion concentrations. We observed that all of the metal oxyanions killed >99% of biofilm and planktonic-cell populations at concentrations slightly in excess of the planktonic MIC. Notably, we observed no precipitation or colorimetric coordination of oxyanions in exposed biofilms treated with the organic chelator sodium diethyldithiocarbamate (Na_2DDTC). This observation would suggest that metal oxyanions may have low affinity for the *E. coli* biofilm matrix and, consequently, that metal sequestration in the EPS may not be a large contributor to the tolerance of biofilms to metal oxyanions. Finally, we tested *E. coli* HM22, a strain that bears a gain-of-function allele of *hipA* that increases the proportion of persisters in stationary-phase populations. Here, we show that multidrug-tolerant persisters from the *HipA7* mutant also have a metal-tolerant phenotype. We thus concluded that the short-term survival of <1% of *E. coli* JM109 populations fits well with the hypothesis that a small population of persister cells may be responsible for the *in vitro* tolerance of both planktonic and biofilm cultures to high concentrations of metal oxyanions.

METHODS

Definitions of measurements. According to the guidelines of the American Clinical Laboratory Standards Institute (CLSI; <http://www.nccls.org/>), the minimum bactericidal concentration (MBC) is defined as the concentration of an antimicrobial agent that kills $3 \log_{10}$ cells of a bacterial culture (or 99.9% of the bacteria). Here, we will denote this measure as $\text{MBC}_{99.9}$. However, the CLSI definition is inadequate for examining the survival of <0.1% of the bacterial population. In this study, we will define MBC_{100} and minimum biofilm-eradication concentration (MBEC) as the concentration of antibiotics or metal(loid) oxyanions required to eradicate 100% of the planktonic and biofilm bacterial populations, respectively. We will use the term 'killing' to denote the death of any proportion of the bacterial population of <100%, and the term 'eradication' will be used to denote death of the bacterial culture (i.e. 100% kill and thus no recoverable viable cells). Lastly, we will define the ratio of $\text{MBEC}:\text{MBC}_{100}$ as the 'fold tolerance' of the biofilm relative to the derived planktonic-cell population. The calculation of fold tolerance is based on data obtained from the MBEC high-throughput (HTP) assay, but not from measurements obtained from the CLSI protocol typically used for antibiotic-susceptibility assays.

Bacterial strains, buffers and media. *E. coli* strains JM109, HM21 and HM22 were stored cryogenically at -70°C in Luria-Bertani (LB) medium containing 8% DMSO. All bacterial cultures were grown in LB medium [pH 7.1; 5 g NaCl, 5 g yeast extract and 10 g tryptone (1 double-distilled water) $^{-1}$] enriched with 0.001% (w/v) vitamin B₁ (LB+B1). Subcultures, MBC_{100} and MBEC determinations and viable cell counts were performed on plates containing LB+B1 medium with 1.5% (w/v) granulated agar. All growth media used for *E. coli* strains HM21 and HM22 were supplemented with 75 μg diamminopimelic acid (DAPA) ml^{-1} . Serial dilutions were carried out with 0.9% saline.

Cultivation of biofilms. Biofilms were grown aerobically in the MBEC-HTP device (MBEC BioProducts) according to the manufacturer's instructions and as described previously (Ceri *et al.*, 1999, 2001). To summarize briefly, there are two parts to this batch-culture apparatus. The top half of the plastic MBEC-HTP device is a lid with 96 pegs that also fits over a standard 96-well microtitre plate. The bottom half is a fluted trough that guides inoculated growth medium across the pegs when the device is placed on a rocker. The rocker provides the shear force that facilitates biofilm formation. Here, 22 ml inoculum (containing $\sim 10^7$ c.f.u. *E. coli* JM109 ml^{-1} suspended in LB+B1 broth) was transferred into the trough. The device was then placed on a rocking table (Bellco Biotechnology) and incubated at 35°C for 24 h and 95% relative humidity at 2.5 rocks min^{-1} . Biofilms formed on the lid of the MBEC-HTP device were rinsed once (by placing the lid in a 96-well microtitre plate with 200 μl 0.9% saline in each well) to remove loosely adherent planktonic bacteria. To verify biofilm formation, four pegs were removed from alternating, parallel rows of the MBEC-HTP device after being rinsed, placed in 200 μl 0.9% saline and sonicated on an Aquasonic water-table sonicator (VWR International, model 250HT) for 5 min on high, as described previously (Ceri *et al.*, 1999, 2001). The disrupted biofilms were diluted serially and plated for viable cell counting.

Antibiotic-susceptibility testing. Amikacin, ceftriaxone and tobramycin (Sigma Chemical) were prepared at 5120 μg ml^{-1} in double-distilled water, passed through a 0.22 μm syringe filter and stored at -70°C until use. This stock solution was diluted tenfold in LB+B1 broth, which served as a working solution to prepare challenge media. Serial twofold dilutions of the working solution were made in LB+B1 broth along the length of a 96-well microtitre plate (resulting in a $\log_2$ concentration gradient from 512 to

1 μg ml^{-1}). The first and last wells of every row were used as sterility and growth controls, respectively. The peg lids (with the rinsed biofilms) were then transferred to the 96-well microtitre plates containing antibiotics. These challenge plates were incubated at 35°C and 95% relative humidity for either 2 or 24 h.

Following exposure, the biofilms were removed from the challenge plates and rinsed twice with 0.9% saline as described above. Biofilm pegs were placed in a 96-well microtitre plate with 200 μl LB+B1 medium in each well and sonicated as described above. Disrupted biofilm cultures from this recovery plate were diluted serially tenfold and plated for viable cell counting. Corresponding planktonic cultures were seeded by bacteria shed from the surface of the mature biofilms (on peg lid of the MBEC-HTP device) into the 96 wells of the antibiotic-challenge plates. Planktonic cells from these cultures were diluted serially tenfold and plated for viable cell counting.

MIC values were obtained by reading OD_{650} of the challenge plates after 56 h incubation at 35°C using a THERMOMax microplate reader with SoftMax Pro data-analysis software (Molecular Devices). Agar plates were incubated for 48 h at 35°C and enumerated to obtain MBC_{100} and MBEC values. MBEC values were redundantly determined (qualitatively) by reading the OD_{650} of the recovery plate after 48 h incubation at 35°C and 95% relative humidity.

Heavy-metal and -metalloid oxyanion-susceptibility testing.

Stock solutions of copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Fisher Scientific), sodium hydrogen arsenate (Na_2HAsO_4) (Fisher Scientific), potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) (J. T. Baker Chemical), selenous acid (H_2SeO_3) (British Drug Houses) and sodium arsenite (NaAsO_2) (Sigma Chemical) were prepared at 40 mg oxyanion ml^{-1} in double-distilled water, passed through a 0.22 μm syringe filter and stored at room temperature until use. Potassium tellurite (K_2TeO_3) (Sigma Chemical) and potassium tellurate (K_2TeO_4) (Johnson Matthey Electronics) have low solubility in water. Tellurite and tellurate were prepared similarly except at concentrations of 10 and 1 mg oxyanion ml^{-1} , respectively.

Biofilm metal-susceptibility testing was performed according to the method of Harrison *et al.* (2004a). This method differed from the method used for antibiotic-susceptibility testing (as outlined above) by virtue of an additional neutralizing protocol. Reduced glutathione (GSH; 5 mM) was used as neutralizing agent for all of the oxyanions examined in this study (Harrison *et al.*, 2004a, b). In the case of biofilm cultures, 5 mM GSH was added directly to the recovery medium before the disruption of biofilms by sonication. In the case of planktonic cells, 40 μl culture was added to 10 μl 25 mM GSH. The neutralized planktonic cultures were incubated for 10 min at room temperature prior to serial dilution. Serially diluted biofilm and planktonic cultures were plated onto LB+B1 agar and incubated for 48 h at 35°C . MIC, MBC_{100} and MBEC values were obtained as described above.

Additional susceptibility testing of exponentially growing planktonic cells.

To validate the MBEC assay used throughout the rest of this study, experiments with exponentially growing *E. coli* JM109 cells were repeated by using a protocol designed to reflect the CLSI method for antibiotic-susceptibility testing. The inoculum for these tests was prepared by direct colony suspension from streak plates to a 1.0 McFarland standard as described above for biofilm cultivation. This standard was diluted 30-fold in LB+B1 medium and 5 μl aliquots of this 1 in 30 dilution were added to each well of the challenge plate. This corresponded to an initial cell load of approximately 10^5 – 10^6 c.f.u. ml^{-1} . Challenge and recovery media, neutralizing agents, serial dilution and incubation times were identical to those described above for biofilm testing.

Susceptibility testing of stationary-phase planktonic cells.

To test directly whether known high-persistence (*hip*) mutants may

have a metal-tolerant phenotype, we examined *E. coli* HM21 (wild-type) and HM22 (*hipA7*). These strains were grown in batch cultures by inoculating 24 ml LB+B1+DAPA with 1 ml 1.0 McFarland standard. These cultures were grown in sterile 50 ml polypropylene tubes for 16 h at 35 °C on a gyratory shaker set to 125 r.p.m. Stationary-phase planktonic cells were harvested by centrifugation (3000 g for 10 min) then suspended in fresh medium containing the appropriate concentration of metal(loid) oxyanions. There was no significant loss of cell viability during centrifugation of either strain (data not shown). Aliquots (20 µl) were removed from the tubes after 6 h exposure, neutralized, diluted serially and plated for viable cell counting as described above. *HipA7* has a temperature-sensitive mutation (Keren *et al.*, 2004b) and, during exposure to metals, these cultures were incubated at 30 °C.

Colorimetric coordination of metal cations and oxyanions in biofilms. Na₂DDTC is an analytical reagent used for determining the concentration of some transition metals, as well as arsenic and tellurium, in aqueous solution (Cheng *et al.*, 1982; Turner *et al.*, 1992). As it pertains to this study, Na₂DDTC forms dark brown-, green- and yellow-coloured complexes with Cu²⁺, CrO₄²⁻ and TeO₃²⁻, respectively. A visible colour change (mediated by Na₂DDTC) of the biofilm pegs in the MBEC-HTP device following metal exposure would indicate that these metal cations and oxyanions had been adsorbed and/or retained by the biofilm matrix (Harrison *et al.*, 2005a). Biofilms were formed on pegs of the MBEC-HTP device (as described above) and then exposed to Cu²⁺, CrO₄²⁻ or TeO₃²⁻ for 24 h. Biofilms exposed to these heavy-metal(loid) cations and oxyanions were rinsed twice for 5 min in sterile 0.9% saline. Subsequently, the peg lid was inserted into a 96-well microtitre plate that contained 200 µl 37.5 mM Na₂DDTC in each well. Biofilms were treated with Na₂DDTC for approximately 2 min, then photographed by using a Sony 4.0 megapixel Cyber-shot digital camera.

Scanning electron microscopy (SEM). Pegs were broken from the lid of the MBEC device and rinsed with 0.9% saline. Biofilms were fixed onto the pegs by incubating at 4 °C overnight in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). The next day, pegs were rinsed with 0.1 M cacodylate buffer, dehydrated with 95% ethanol and then air-dried for 30 h before mounting. SEM was performed by using a Hitachi model 450 scanning electron microscope as described by Morck *et al.* (1994).

RESULTS

Biofilm formation

Biofilms were grown on the peg lid of the MBEC-HTP assay to a point that corresponded to mid-stationary phase for the associated planktonic cells in the trough of the device. Data from 54 control assays were pooled to calculate the population mean ± SD of biofilm growth on the pegs. At 24 h, biofilms had a mean cell density of 3.6 ± 2.9 (× 10⁶) c.f.u. per peg in the MBEC device.

SEM was used to examine biofilm formation *in situ*. SEM photomicrographs of *E. coli* JM109 are presented in Supplementary Fig. S1 (available with the online version of this paper). Consistent with previous reports using this method (Ceri *et al.*, 1999; Olson *et al.*, 2002; Harrison *et al.*, 2004a), these pictures show the growth of mature biofilms on the peg surface.

Time-dependent susceptibility of *E. coli* to antibiotics

As a standard of comparison to metal(loid) oxyanions and as a quality control for our experimental system, we examined the growth inhibition and eradication of *E. coli* JM109 by three different antibiotics: amikacin, ceftriaxone and tobramycin. The means ± SD for observed MIC, MBC₁₀₀ and MBEC values are summarized in Table 1. Each reported value is based on four to eight independent replicates, and MBEC values obtained from (qualitative) OD₆₅₀ measurements of recovery plates were also included in this calculation. In no instance did antibiotics eradicate biofilm cultures of *E. coli* with 2 h exposure. By 24 h, planktonic cultures were eradicated at relatively low concentrations of amikacin (20 ± 8 µg ml⁻¹), ceftriaxone (88 ± 48 µg ml⁻¹) and tobramycin (36 ± 20 µg ml⁻¹). Regardless of exposure time, biofilms could not be eradicated at the highest concentrations of amikacin or ceftriaxone examined (512 µg ml⁻¹). Tobramycin eradicated biofilms in a time-dependent manner at very high concentrations (352 ± 192 µg ml⁻¹). However, these assays show that *E. coli* biofilms remained at least 9.8–51 times more tolerant to antibiotics than planktonic cells (even with prolonged exposure time). This observation is consistent with previously reported results (Ceri *et al.*, 1999; Walters *et al.*, 2003; Harrison *et al.*, 2004a).

Log killing of *E. coli* biofilms by antibiotics

The bactericidal action of amikacin, ceftriaxone and tobramycin with respect to antibiotic concentration was evaluated by determining the viable cell counts of *E. coli* JM109 after 2 or 24 h exposure. Mean viable cell counts and log killing for biofilm cultures are illustrated in Fig. 1. Each value is reported as the mean ± SD of four independent replicates. The majority of biofilm and planktonic populations were eradicated at very low concentrations of

Table 1. Time-dependent susceptibility of *E. coli* JM109 to antibiotics

NA, Not applicable; bold type indicates fold tolerance at 24 h exposure.

Antibiotic	MIC (µg ml ⁻¹)	MBC ₁₀₀ (µg ml ⁻¹)	MBEC (µg ml ⁻¹)	Fold tolerance*
Amikacin				
2 h	2.5 ± 1	> 512	> 512	NA
24 h	8 ± 6	20 ± 8	> 512	≥ 51
Ceftriaxone				
2 h	6 ± 2	> 512	> 512	NA
24 h	30 ± 25	88 ± 48	> 512	≥ 12
Tobramycin				
2 h	15 ± 12	36 ± 20	> 512	≥ 28
24 h	24 ± 9	36 ± 20	352 ± 192	9.8

*Fold tolerance, given the sensitivity of the assay on a log₂ scale, is equal to the ratio of the mean MBEC to the mean MBC₁₀₀.

Fig. 1. Killing of *E. coli* JM109 biofilms by antibiotics. Biofilms were grown for 24 h on the peg lid of the MBEC-HTP device, then exposed for 2 or 24 h to amikacin, ceftriaxone or tobramycin in LB+B1 medium. Biofilm and planktonic cultures were recovered in fresh LB+B1 medium, diluted serially tenfold, then plated onto agar for viable cell counts. Each data point is the mean of three or four independent replicates; error bars denote SD. (a, c, e) Mean viable cell counts for planktonic and biofilm cultures with respect to concentration of antibiotic. (b, d, f) Log killing of biofilm cultures by antibiotics. Low concentrations of antibiotics destroyed the majority of planktonic and biofilm populations rapidly. A very small fraction of the population (10^{-3} to 10^{-6}) survived exposure to the highest concentrations of amikacin and ceftriaxone tested ($512 \mu\text{g ml}^{-1}$). In the case of planktonic cultures, these 'persisters' were eradicated by 24 h exposure. Only tobramycin could effectively eradicate biofilms with 24 h exposure – in this case, at the threshold of the highest concentration assayed ($128 \mu\text{g ml}^{-1}$). $\blacktriangle$ and $\triangle$ denote biofilm cultures at 2 and 24 h exposure, respectively; $\blacksquare$ and $\square$ denote planktonic cultures at 2 and 24 h exposure, respectively; an asterisk denotes a concentration at which the culture was eradicated; the starting concentration of antibiotic after the break in the x axis is the same as the final concentration before the break.

amikacin (approx. $20 \mu\text{g ml}^{-1}$). However, 0.1 % or less of the population tolerated this antibiotic up to $512 \mu\text{g}$

amikacin ml^{-1} , which was the highest concentration used in this assay. A similar trend was observed for ceftriaxone. In

the case of planktonic cultures, the surviving cells were eradicated in a time-dependent manner (Fig. 1a), a trait that has been attributed to persister cells (Balaban *et al.*, 2004). When the bacteria were grown in a biofilm, a small fraction of cells were recalcitrant to killing by amikacin and ceftriaxone, even with 24 h exposure (Fig. 1b and d). Tobramycin showed time-dependent killing kinetics of *E. coli* biofilms (Fig. 1e and f). At 24 h exposure, tobramycin was the only antibiotic to eradicate biofilms and this occurred at the threshold of the highest concentration examined ($352 \pm 192 \mu\text{g ml}^{-1}$). These results are consistent with previous reports in the literature that have examined antibiotic susceptibility of *P. aeruginosa* biofilms (Spoering & Lewis, 2001). In general, our data show that, although biofilms may be killed in a time-dependent manner by antibiotics, they are many times more resilient to these drugs than planktonic cells (even with long exposures).

Time-dependent susceptibility of *E. coli* to heavy-metal(loid) oxyanions

E. coli JM109 MIC, MBC₁₀₀ and MBEC values for CrO_4^{2-} , AsO_4^{3-} , AsO_2^- , SeO_3^{2-} , TeO_4^{2-} and TeO_3^{2-} at 2 and 24 h

exposure are presented in Table 2. Each value is reported as the mean $\pm$ SD of three to seven independent replicates, and MBEC values obtained from OD₆₅₀ measurements of recovery plates were also included in this calculation. With short exposures (i.e. 2 h), *E. coli* biofilms were tolerant to very high concentrations of metal oxyanions. In the case of TeO_3^{2-} , biofilm cultures presented with a transient 310-fold increased tolerance relative to the corresponding planktonic cultures. The bacterial survivors of tellurite exposure represented a small-enough proportion of the population that they were only detected reliably by using the (qualitative) OD₆₅₀ measurements of the 96-well microtitre recovery plate. This may imply that the number of surviving cells in the biofilm was approximately equal to or less than the threshold of detection using viable cell counts (approx. 10 c.f.u. per peg). A similar phenomenon was observed for CrO_4^{2-} . In all instances, the highly tolerant survivors were eradicated by metal oxyanions by 24 h exposure. As a comparison to literature values, the MIC values obtained by using the MBEC-HTP method for SeO_3^{2-} and TeO_3^{2-} are approximately equal to the MIC values obtained by using alternative microbiological techniques (Turner *et al.*, 1998; Turner, 2001).

Table 2. Susceptibility of *E. coli* JM109 to heavy-metal and heavy-metalloid oxyanions with 2 or 24 h exposure

NA, Not applicable; ND, not determined; bold type indicates fold tolerance at 24 h exposure.

Metal ion	CLSI assay		MBEC-HTP assay			Fold tolerance*
	MIC (mM)	MBC ₁₀₀ (mM)	MIC (mM)	MBC ₁₀₀ (mM)	MBEC (mM)	
CrO_4^{2-}						
2 h	0.54 ± 0	1.8 ± 0.6	0.78 ± 0.40	8.8 ± 0	26 ± 10	3.0
24 h		0.44 ± 0.16		0.55 ± 0	0.55 ± 0	1.0
AsO_4^{3-}						
2 h	ND	> 77	26 ± 24	> 77	> 77	NA
24 h		> 77		> 77	> 77	NA
AsO_2^-						
2 h	9.4 ± 0	> 75	3.8 ± 1.3	> 59	> 59	NA
24 h		75 ± 0		59 ± 0	59 ± 0	1.0
SeO_3^{2-}						
2 h	8.1 ± 0	64 ± 0	9.2 ± 3.0	32 ± 0	56 ± 16	1.8
24 h		13 ± 5		8.1 ± 0	13 ± 5	1.6
TeO_4^{2-}						
2 h	ND	1.4 ± 0	6.5 ± 1.6 ($\times 10^{-2}$)	1.6 ± 0.6 †	> 1.1	≥ 2.0
24 h		1.1 ± 0.4				
TeO_3^{2-}						
2 h	1.8 ± 0.7 ($\times 10^{-3}$)	0.03 ± 0.01	1.0 ± 0.2 ($\times 10^{-2}$)	0.03 ± 0.01	8.7 ± 3.4	3.1×10^2
24 h		9.5 ± 3.2 ($\times 10^{-3}$)		6.6 ± 4.3 ($\times 10^{-3}$)	(3.3 ± 2.2) ($\times 10^{-3}$)	0.50

*Fold tolerance, given the sensitivity of the assay on a log₂ scale, is equal to the ratio of the mean MBEC to the mean MBC₁₀₀.

†Denotes that eradication of the indicated culture occurred at the threshold of the highest concentration of metal tested.

As a quality control for the MBEC experimental system, planktonic-cell susceptibility testing was repeated with exponentially growing planktonic cells prepared in a manner similar to a CLSI protocol used for antibiotic-susceptibility testing. In these assays, the mean initial cell load was similar to the biofilm cell density per peg of the MBEC-HTP assay (see below). MIC and MBC_{100} values obtained by using this method are presented in Table 2. These values were similar and consistent with those obtained by using the MBEC-HTP assay. Each value represents the mean $\pm$ SD of four independent trials.

Log killing of *E. coli* biofilms by heavy-metal(loid) oxyanions

To examine the survival of planktonic and biofilm bacterial populations following exposure to metal(loid) oxyanions, viable cell counts were determined for a range of concentrations following either 2 or 24 h exposure. Mean viable cell counts and log killing of biofilm cultures with respect to concentration of CrO_4^{2-} , AsO_4^{3-} and AsO_2^- are presented in Fig. 3. The data for SeO_3^{2-} , TeO_4^{2-} and TeO_3^{2-} are presented in Fig. 4. Every metal oxyanion examined in this study killed a large proportion ($2\log_{10}$ cells or greater) of the biofilm at relatively low concentrations. To continue with the example of tellurite, the majority of both planktonic and biofilm populations were killed at $7\text{ }\mu\text{M}$ TeO_3^{2-} (approx. $1\text{--}2\text{ }\mu\text{g ml}^{-1}$). However, a small fraction of biofilm cells (10^{-5} to 10^{-3}) survived for at least 2 h at concentrations well in excess of 0.1 mM ($16\text{--}32\text{ }\mu\text{g ml}^{-1}$). In addition to the data from Table 2, a very small number of survivors (below the threshold of detection by viable cell counts) tolerated TeO_3^{2-} at concentrations up to 12 mM (approx. $2048\text{ }\mu\text{g ml}^{-1}$). This surviving fraction was eradicated by 24 h exposure. Only AsO_4^{3-} did not eradicate biofilm or planktonic cultures with 24 h exposure, and only 10^{-3} c.f.u. biofilm per peg survived this toxic exposure. High concentrations of CrO_4^{2-} , AsO_2^- , SeO_3^{2-} , TeO_4^{2-} and TeO_3^{2-} eradicated biofilm and planktonic cultures completely with extended exposure times.

There are two features of the MBEC-HTP assay that must be addressed here. First, the wells of the microtitre plates containing serial dilutions of metals are inoculated with bacteria shed from the surface of the peg lid. As a consequence, the precise initial number of planktonic bacteria is unknown, and log killing of planktonic cells cannot be calculated by using this method. However, this situation *in vitro* may be reflective of naturally existing environmental systems (or as a model of infection) where a biofilm forms a recalcitrant nucleus that sheds planktonic cells into its surroundings.

Second (and critical to the seeding of planktonic wells), transfer of the peg lid from the MBEC-HTP device into challenge medium inherently results in the dispersion of a proportion of the *E. coli* JM109 biofilm. We have noted previously that, for *P. aeruginosa* ATCC 27853 biofilms, this dispersion event is not statistically significant (J. J. Harrison,

R. J. Turner & H. Ceri, unpublished data). However, the proportion of the *E. coli* JM109 biofilm that is disrupted is larger. In the absence of shear and given sufficient exposure time, the disrupted biofilms resume growing. Here, this works as an advantage. Mean viable cell counts reported in Figs 1, 2 and 3 were based on the number of bacteria recovered from pegs (including growth controls) after transfer and exposure. However, log killing was calculated relative to the initial number of bacteria formed in the biofilm before transfer to the challenge medium (i.e. log killing was based on the growth controls used to calculate the mean biofilm cell density of 3.6 ± 2.9 c.f.u. per peg as described above). We can thus discern that growth of biofilms occurs during exposure to low concentrations of metal(loid) oxyanions. If we compare where the transition between growth and cell death occurs on the log-killing curves in Fig. 3(b, d, f) and Fig. 4(b, d, f), biofilms were not observed to grow at concentrations of metal(loid) oxyanions greater than the planktonic MIC values reported in Tables 1 and 2. As noted above, the MIC values in Tables 1 and 2 are consistent with literature MIC values and those obtained by using a modified CLSI protocol (Table 2). This results in the interesting notion that biofilms and planktonic cells may be similarly resistant to growth inhibition by metal(loid) oxyanions.

Log killing of exponentially growing planktonic cells by metal(loid) oxyanions using alternative methods

When comparing the resistance and/or tolerance of planktonic cells and biofilms to metals, there is a possibility that differences in susceptibility may arise due to disparity in starting cell numbers used for MIC, MBC and MBEC determinations. To check for this possibility, we chose a representative compound from each periodic group of metals and repeated cell-viability testing by using a protocol similar to CLSI guidelines for antibiotic-susceptibility assays. The means $\pm$ SD of viable cell counts were determined for chromate, arsenite and tellurite and are reported in Fig. 4. The initial cell load using this complementary method was 9.7 ± 7.6 ($\times 10^5$) c.f.u. ml^{-1} and log killing of planktonic cultures was calculated based on this number. This starting cell number was similar to the starting cell densities of the biofilms tested in the preceding sections (10^6 cells). MBC values obtained by using this alternative method were generally within $\log_2$ of the values obtained by using the MBEC-HTP assay. Notably, the proportion of surviving cells was smaller than the fraction of survivors recovered from biofilms. These data serve as an important quality control for making comparisons between the susceptibility of planktonic cells and biofilms to metals by using the MBEC device.

Retention and reduction of metal(loid) oxyanions by *E. coli* biofilms

We have observed previously that biofilms of *P. aeruginosa* ATCC 27853 adsorb cupric cations (Cu^{2+}) (Harrison *et al.*,

Fig. 2. Killing of *E. coli* JM109 biofilms by chromium and arsenic oxyanions. Biofilms were exposed to CrO_4^{2-} , AsO_4^{3-} or AsO_2^- for 2 or 24 h. (a, c, e) Log_{10} -transformed mean viable cell counts for biofilm and planktonic cultures correlative to concentration of CrO_4^{2-} , AsO_4^{3-} or AsO_2^- , respectively. (b, d, f) Log killing of corresponding biofilm cultures. Experimental conditions and data analysis are identical to those described in the legend to Fig. 1. In the case of arsenic oxyanions, concentrations in excess of approximately 5 mM resulted in the death of 99.9% (or a greater proportion) of the bacterial culture. The surviving 0.1% of the population tolerated these highly toxic heavy metalloids at concentrations in excess of 50 mM. AsO_4^{3-} did not eradicate these surviving populations, even at the highest concentration examined (59 mM). Chromium (VI) began to kill bacteria at concentrations as low as 400 μM ; however, 0.1% of the bacterial population survived for at least 2 h at concentrations of up to 8.8 mM. Notably, CrO_4^{2-} and AsO_2^- eradicated biofilm cultures in a time-dependent manner. $\blacktriangle$ and $\triangle$ denote biofilm cultures at 2 and 24 h exposure, respectively; $\blacksquare$ and $\square$ denote planktonic cultures at 2 and 24 h exposure, respectively; an asterisk denotes a concentration at which the culture was eradicated; the starting concentration of oxyanion after the break in the x axis is the same as the final concentration before the break.

Fig. 3. Killing of *E. coli* JM109 biofilms by selenium and tellurium oxyanions. Biofilms were exposed to SeO_3^{2-} , TeO_4^{2-} or TeO_3^{2-} for 2 or 24 h. (a, c, e) Mean viable cell counts for biofilm and planktonic cultures correlative to concentration of SeO_3^{2-} , TeO_4^{2-} or TeO_3^{2-} , respectively. (b, d, f) Log killing of corresponding biofilm cultures. Experimental conditions and data analysis are identical to those described in the legend to Fig. 2. Consistently, only a small percentage of the bacterial population (approx. 0.1 % or less) was observed to survive at high concentrations of these highly toxic metalloids oxyanions. In the case of biofilm cultures, the surviving subpopulation was eradicated in a time-dependent manner. In other words, biofilm and planktonic cultures were equally susceptible to these compounds with extended exposure times. $\blacktriangle$ and $\triangle$ denote biofilm cultures at 2 and 24 h exposure, respectively; $\blacksquare$ and $\square$ denote planktonic cultures at 2 and 24 h exposure, respectively; an asterisk denotes a concentration at which the culture was eradicated; the starting concentration of oxyanion after the break in the x axis is the same as the final concentration before the break.

Fig. 4. Killing of *E. coli* JM109 exponentially growing planktonic cells by chromate (CrO_4^{2-}), arsenite (AsO_2^-) and tellurite (TeO_3^{2-}). (a, c, e) Mean viable cell counts for planktonic cultures correlative to concentration of CrO_4^{2-} , AsO_2^- or TeO_3^{2-} , respectively. (b, d, f) Log killing of corresponding planktonic cultures. These assays were performed by using a protocol designed to reflect a CLSI assay for antibiotic-susceptibility testing. Very small proportions (i.e. 10^{-3} to 10^{-5}) of planktonic-cell populations survived exposure to bactericidal concentrations of these metal oxyanions. This proportion of surviving cells was smaller than the fraction of survivors recovered from biofilms. Similar to control antibiotic assays, planktonic cells were eradicated in a time-dependent manner by metal oxyanions. These kill curves are similar to those obtained by using the MBEC-HTP assay. However, log killing of planktonic cells by metals may be calculated by using this method, as the starting cell number is known. ■ and □ denote planktonic cultures at 2 and 24 h exposure, respectively; an asterisk denotes a concentration at which the culture was eradicated; the starting concentration of oxyanion after the break in the x axis is the same as the final concentration before the break.

2005a). We thus addressed the question of whether the observed time-dependent killing of *E. coli* JM109 may be partly due to oxyanion adsorption to the biofilm matrix. As a control, we exposed biofilms of *E. coli* JM109 to Cu^{2+} for 24 h, rinsed the biofilms with 0.9 % saline, then treated the exposed biofilms with the chelator Na_2DDTC . Correlative with our previous observations, Na_2DDTC caused the formation of dark-brown metal chelates in the biofilm (Fig. 5a, d, g). However, Na_2DDTC did not cause the characteristic colorimetric coordination of oxyanions in biofilms exposed to either CrO_4^{2-} (Fig. 5b, e, h) or TeO_3^{2-} (Fig. 5c, f, i). Surprisingly, this would suggest that EPS adsorption of oxyanions may not be the primary mechanism of the observed time-dependent tolerance of biofilms to either CrO_4^{2-} or TeO_3^{2-} .

Of note, the reduction of periodic group 6A oxyanions SeO_3^{2-} (data not shown) and TeO_3^{2-} (Fig. 5c and i) to orange and grey-black end products (respectively) was mediated by both planktonic and biofilm cultures of *E. coli* JM109. For planktonic cultures exposed to TeO_3^{2-} , this was observed as grey discoloration in the challenge medium after 24 h exposure (Fig. 5c). In biofilm cultures, grey-black

bands could be observed at the biofilm–air–liquid interface (Fig. 5i).

Survival of the *E. coli* *hipA7* mutant after exposure to metals

To test directly whether persisters are responsible for bacterial tolerance to metal(loid) oxyanions, a *hipA7* strain of *E. coli* was examined. *hipA7* is a gain-of-function allele of *hipA* that increases the proportion of persisters in biofilms and stationary-phase planktonic cultures (Keren *et al.*, 2004b). Stationary-phase *E. coli* HM21 (wild-type) and HM22 (*hipA7*) were exposed to bactericidal concentrations of TeO_3^{2-} (1.5 mM), TeO_4^{2-} (1.3 mM) and CrO_4^{2-} (2.2 mM) for 6 h, then enumerated. Relative to the wild-type strain, *E. coli* HM22 (*hipA7*) had an 82-fold-, 76-fold- and fivefold-increased proportion of survivors after exposure to these metal(loid) oxyanions, respectively. These data are summarized in Supplementary Fig. S2 (available with the online version of this paper). At the highest concentrations of arsenate, arsenite and selenite tested against *E. coli* JM109, there was insufficient killing of *E. coli* HM21 and HM22 at 6 h exposure to observe a hypertolerant

	Cu^{2+}		CrO_4^{2-}		TeO_3^{2-}	
Planktonic
Planktonic + Na_2DDTC
Biofilm + Na_2DDTC
Conc.	control	130 $\longrightarrow$ 8.1 mM	control	18 $\longrightarrow$ 1.1 mM	control	12 $\longrightarrow$ 0.75 mM

Fig. 5. Metal oxyanions were not retained in the biofilm matrix of *E. coli* JM109. Planktonic cultures of *E. coli* JM109 were exposed to Cu^{2+} (a), CrO_4^{2-} (b) or TeO_3^{2-} (c) for 24 h, then reacted with 37.5 mM Na_2DDTC (an organic chelator). Complexation of Cu^{2+} , CrO_4^{2-} and TeO_3^{2-} with Na_2DDTC resulted in an instantaneous colour change of planktonic cultures to dark brown (d), green (e) and bright yellow (f), respectively. Biofilms exposed to Cu^{2+} also underwent a colour change (g), indicating that Cu^{2+} may be retained within the biofilm matrix. In contrast, CrO_4^{2-} (h)- and TeO_3^{2-} (i)-exposed biofilms treated with Na_2DDTC did not change to a colour characteristic of the metal–chelator complex. With 24 h exposure to TeO_3^{2-} , both biofilm and planktonic cultures turned grey-black, a hallmark of bacterial-mediated tellurite reduction. Collectively, these observations suggest that negatively charged metal oxyanions may have low affinity for the biofilm matrix. The left-hand well of every panel is a sterility control. Growth controls not exposed to metals (not shown) did not change colour in any assay.

phenotype for the *hipA7* mutant (data not shown). The starting numbers of cells for *E. coli* HM21 and HM22 were $5.3 \pm 0.6 (\times 10^8)$ and $1.2 \pm 0.3 (\times 10^8)$ c.f.u. ml⁻¹, respectively. *E. coli* HM22 entered the stationary phase at lower cell densities than *E. coli* HM21 (data not shown).

DISCUSSION

In this study, we used an HTP technique (the MBEC-HTP assay) to screen the time- and concentration-dependent toxicity of metal oxyanions on biofilm and planktonic cells of *E. coli* JM109. This bacterium is a laboratory strain with a history of use in studies of metal resistance. The MBEC-HTP assay provided three internally consistent measurements from a single experiment: the planktonic MIC, the planktonic MBC₁₀₀ and the MBEC. In this assay, planktonic cells used in susceptibility testing were shed from the surface of biofilm, reproducing *in vitro* the characteristic duality of the natural bacterial life cycle. This feature is unique to the method used here. As a control for this experimental system, MIC and MBC₁₀₀ values attained by using this technique were compared with those discerned by using a modified CLSI protocol. Initial cell numbers used in these additional assays were similar to biofilm cell densities in the MBEC device. The MIC and MBC₁₀₀ values obtained from both methods were similar (serving as an important quality control for our experimental system). Subsequently, combinations of exposure times and metal concentrations were examined rapidly by using the MBEC-HTP assay, which would not have been pragmatic using alternative techniques. To validate this model, we have re-examined the biofilm persister-cell hypothesis proposed by Spoering & Lewis (2001). Consistent with previously reported results, we observed here that biofilms of *E. coli* remained highly tolerant to antibiotics for long exposure times (Walters *et al.*, 2003; Harrison *et al.*, 2004a). Further, this tolerance was mediated by a very small proportion of the adherent bacterial population (Spoering & Lewis, 2001).

E. coli HM22 (*hipA7*) – well-known for its ability to produce a large proportion of persisters recalcitrant to killing by bactericidal antibiotics – also produces a subpopulation of metal-tolerant cells that is larger than the population of the isogenic, wild-type strain. Biofilms of *E. coli* JM109 presented with a transient, time-dependent tolerance to metal oxyanions that was up to two orders of magnitude greater than that of planktonic cells. Long exposures to these highly toxic substances resulted in destruction of planktonic and biofilm cultures at similar concentrations of metal oxyanions. Consistent with the persister-cell hypothesis, this short-term tolerance was mediated by a small fraction of the biofilm population (10^{-3} or less). There are two potential explanations for this phenomenon: (i) that persister cells in a biofilm are killed at a reduced rate by metal oxyanions relative to the planktonic persister population, or (ii) that there is a greater population of persisters in a biofilm that are killed at the same rate as planktonic persister cells. In either case, this is different from antibiotic

killing of biofilms, where cell death typically reached a plateau characterized by a lack of further antibiotic efficacy (even with prolonged exposure or increased antibiotic concentrations). In the case of tobramycin, which did eradicate biofilms by 24 h exposure, biofilms remained 9.8 times more tolerant to killing than the corresponding planktonic cells. Thus, our data would suggest that metal oxyanions possess a bactericidal action capable of destroying biofilm persisters. To reaffirm this notion, we have observed in a subsequent study that stationary-phase *E. coli* HM22 may be eradicated by arsenate with 24 h exposure (J. J. Harrison, R. J. Turner & H. Ceri, unpublished data).

The role of *E. coli* EPS in adsorption of metal oxyanions had previously been unexamined. Here, metal oxyanions may have equilibrated across the biofilm matrix at a slowed rate due to steric and/or ionic hindrance (Stewart, 2003). Intuitively, we can be confident that complete penetration of the EPS matrix eventually occurred if the biofilms were eradicated. The observation that the chelator Na₂DDTC does not result in precipitation or coordination of metal oxyanions in exposed biofilms supports the idea that the *E. coli* JM109 EPS matrix may have low affinity for adsorbing and/or binding metal oxyanions. We noted that this was not the case for the divalent heavy-metal cation Cu²⁺, which was retained and formed metal precipitates in similarly treated biofilm cultures. Our observations are consistent with reports that the *E. coli* EPS is polyanionic (reviewed by Sutherland, 2001). Colanic acid, which is known to be important in *E. coli* biofilm formation (Potrykus & Węgrzyn, 2004), is the major extracellular polysaccharide of most *E. coli* strains (Gottesman & Stout, 1991; Whitfield & Roberts, 1999). Further evidence for the low affinity of the matrix for metal oxyanions was the observed reduction of tellurite (Fig. 5i) by biofilm cultures.

The reduction of tellurite by *E. coli* has been described previously (reviewed by Turner, 2001). Reduction of TeO₃²⁻ to grey-black-coloured, colloidal Te⁰ is an intracellular reaction potentially mediated by the terminal cytochrome *c* oxidase of the respiratory electron-transfer chain (Trutko *et al.*, 2000) and/or through an intermediate Painter reaction of TeO₃²⁻ with glutathione (Turner *et al.*, 2001). Insoluble Te⁰ associates closely with cell membranes (Lloyd-Jones *et al.*, 1994), giving bacterial cultures a characteristic grey-black colour. The observation that *E. coli* biofilms turn grey-black after exposure to TeO₃²⁻ (Fig. 5i) is evidence of intracellular Te accumulation and entrapment. This is evidence that the biofilm matrix may not sequester metal oxyanions in the extracellular milieu. A similar case can be made for selenite reduction, another group 6A chalcogen with similar chemistry to tellurite (reviewed by Turner *et al.*, 1998). In the present study, *E. coli* biofilm cultures also reduced SeO₃²⁻ to Se⁰, which resulted in the accumulation of orange-red end products on the pegs (data not shown), similar to *P. aeruginosa* (Harrison *et al.*, 2004b).

We have recently conducted studies examining the toxicity of metal cations (in both *E. coli* JM109 and *P. aeruginosa*

ATCC 27853) to further validate the persister-cell phenomenon reported here for metal oxyanions (Harrison *et al.*, 2005a, b). A characteristic attributed to persisters is the ability of these survivors to give rise to a new population with normal susceptibility (Bigger, 1944; Moyed & Bertrand, 1983; Spoering & Lewis, 2001; Keren *et al.*, 2004a). We have thus addressed this question for *E. coli* JM109. Persisters cultivated after exposure to mercury (Hg^{2+}), then to copper (Cu^{2+}) and finally to Cu^{2+} again yielded a similar proportion of metal persisters in the newly generated populations and produced cultures with normal susceptibility to metals (J. J. Harrison, H. Ceri & R. J. Turner, unpublished data). A similar phenomenon occurred for *P. aeruginosa* ATCC 27853 (Harrison *et al.*, 2005a). Collectively, this would indicate that persisters are not mutants within the bacterial culture, but rather specialized survivor cells produced by a genetically homogeneous population.

In *E. coli*, the proportion of persisters in the bacterial population is in part controlled by the high-persistence (*hip*) operon (Moyed & Bertrand, 1983; Korch *et al.*, 2003; Keren *et al.*, 2004b). The *hipA7* mutant produces 10- to 1000-times more persister cells than wild-type *E. coli* as it approaches stationary phase (Korch *et al.*, 2003; Keren *et al.*, 2004b). As the *hipA7*-mediated high-persistence phenotype was abolished in a *relA*[−] *spoT*[−] background, Korch *et al.* (2003) postulated that (p)ppGpp synthesis may govern the 'persistent' physiological state. More than 80 homologues of the *hipA* gene have been identified in Gram-negative and Gram-positive bacteria (Korch *et al.*, 2003). It is unknown what role the stringent response [which is also governed by (p)ppGpp synthesis] plays in the production of persisters. However, it is known that stringent response-regulated genes play an important role in *E. coli* biofilm formation (Balzer & McLean, 2002), entry into stationary phase, bacterial adaptation to nutrient limitation and oxidative stress (Chang *et al.*, 2002). The 'persistent' response of *hip* mutants not only reduces cell death due to antibiotic exposure, but also reduces the lethality of heat shock (Scherrer & Moyed, 1988) and metabolic block (i.e. starvation for DAPA) (Moyed & Bertrand, 1983). We acknowledge that some of these mechanisms may share overlapping features with each other and the effects of metal toxicity.

Metal oxyanions are known to exert toxicity through oxidative stress (Stohs & Bagchi, 1995; Turner *et al.*, 1998, 1999). Additionally, arsenate is toxic by virtue of substitution for inorganic phosphate in glycolytic intermediates, abrogating ATP-producing enzymic steps through spontaneous hydrolysis of arsenophosphates in water (reviewed by Moran *et al.*, 1994). Although the precise mechanism is unknown, tellurite ultimately functions to uncouple the *E. coli* transmembrane ΔpH gradient (Lohmeier-Vogel *et al.*, 2004). In both cases, ATP levels would be lowered in sensitive cells, mimicking the effects of starvation. Thus, the data in this study and in the literature suggest that persistence may be part of an underlying, highly conserved and generalized mechanism for bacteria to tolerate

environmental duress. Within the limits of our current understanding, persisters may be defined phenotypically as the small, slow-growing and physiologically distinct sub-population of bacterial cells capable of withstanding stress (Balaban *et al.*, 2004; Keren *et al.*, 2004b).

Biofilms are infamous for their ability to withstand antimicrobials. Here, we noted that biofilms did not grow at concentrations of metal oxyanions in excess of the planktonic MIC. Based on our data, we may not label bacterial biofilms as 'resistant'. However, biofilms did withstand concentrations of metal oxyanions far in excess of the planktonic MBC₁₀₀. Biofilms may thus be considered highly 'tolerant' to metal oxyanions. Persisters are known to survive high levels of antibiotics for prolonged exposure times. As it pertains to our model system and *E. coli* JM109, this is not true for metal oxyanions. In fact, given long exposures, metal oxyanions destroyed biofilm cultures at concentrations similar to those that eradicated planktonic bacteria. In this study, we observed that 1 % or less of the biofilm population survived for short periods of time at concentrations of metal cations in excess of the concentration required to eradicate planktonic bacteria (MBC₁₀₀). We thus propose that persister cells mediate the time-dependent tolerance of *E. coli* biofilms to metal oxyanions and, further, that these compounds may be lethal to the slow-growth phenotype possessed by persister cells.

ACKNOWLEDGEMENTS

This work was supported by Natural Science and Engineering Research Council of Canada (NSERC) grants to R. J. T. and H. C. NSERC has provided a summer studentship for E. A. B. and an Industrial Postgraduate Scholarship (IPS1) to J. J. H. Thanks to Dr Kim Lewis for kindly providing *E. coli* strains HM21 and HM22. Additional thanks go to Liz Middlemiss for her technical expertise with SEM.

REFERENCES

- Balaban, N. Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. (2004). Bacterial persistence as a phenotypic switch. *Science* **305**, 1622–1625.
- Balzer, G. J. & McLean, R. J. C. (2002). The stringent response genes *relA* and *spoT* are important for *Escherichia coli* biofilms under slow-growth conditions. *Can J Microbiol* **48**, 675–680.
- Beloin, C., Valle, J., Latour-Lambert, P. & 8 other authors (2004). Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. *Mol Microbiol* **51**, 659–674.
- Bigger, J. W. (1944). Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* **ii**, 497–500.
- Brooun, A., Liu, S. & Lewis, K. (2000). A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* **44**, 640–646.
- Brown, G. E., Jr, Foster, A. L. & Ostergren, J. D. (1999). Mineral surfaces and bioavailability of heavy metals: a molecular-scale perspective. *Proc Natl Acad Sci U S A* **96**, 3388–3395.
- Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. & Buret, A. (1999). The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J Clin Microbiol* **37**, 1771–1776.

- Ceri, H., Olson, M., Morck, D., Storey, D., Read, R., Buret, A. & Olson, B. (2001). The MBEC assay system: multiple equivalent biofilms for antibiotic and biocide susceptibility testing. *Methods Enzymol* **337**, 377–384.
- Cervantes, C., Campos-García, J., Devars, S., Gutiérrez-Corona, F., Loza-Tavera, H., Torres-Guzmán, J. C. & Moreno-Sánchez, R. (2001). Interactions of chromium with microorganisms and plants. *FEMS Microbiol Rev* **25**, 335–347.
- Chang, D.-E., Smalley, D. J. & Conway, T. (2002). Gene expression profiling of *Escherichia coli* growth transitions: an expanded stringent response model. *Mol Microbiol* **45**, 289–306.
- Chasteen, T. G. & Bentley, R. (2003). Biomethylation of selenium and tellurium: microorganisms and plants. *Chem Rev* **103**, 1–25.
- Cheng, K. L., Ueno, K. & Imamura, T. (1982). *CRC Handbook of Organic Analytical Reagents*. Boca Raton, FL: CRC Press.
- Donlan, R. M. & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167–193.
- Falla, T. J. & Chopra, I. (1998). Joint tolerance to β -lactam and fluoroquinolone antibiotics in *Escherichia coli* results from over-expression of *hipA*. *Antimicrob Agents Chemother* **42**, 3282–3284.
- Geslin, C., Llanos, J., Prieur, D. & Jeanthon, C. (2001). The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. *Res Microbiol* **152**, 901–905.
- Gottesman, S. & Stout, V. (1991). Regulation of capsular polysaccharide synthesis in *Escherichia coli* K12. *Mol Microbiol* **5**, 1599–1606.
- Hall-Stoodley, L., Costerton, J. W. & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nat Rev Microbiol* **2**, 95–108.
- Harrison, J. J., Ceri, H., Stremick, C. A. & Turner, R. J. (2004a). Biofilm susceptibility to metal toxicity. *Environ Microbiol* **6**, 1220–1227.
- Harrison, J. J., Ceri, H., Stremick, C. & Turner, R. J. (2004b). Differences in biofilm and planktonic cell mediated reduction of metalloid oxyanions. *FEMS Microbiol Lett* **235**, 357–362.
- Harrison, J. J., Turner, R. J. & Ceri, H. (2005a). Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic *Pseudomonas aeruginosa*. *Environ Microbiol* **7**, 981–994.
- Harrison, J. J., Turner, R. J. & Ceri, H. (2005b). Metal tolerance in bacterial biofilms. *Recent Res Dev Microbiol* **9**, 33–55.
- Huang, C.-T., Xu, K. D., McFeters, G. A. & Stewart, P. S. (1998). Spatial patterns of alkaline phosphatase expression within bacterial colonies and biofilms in response to phosphate starvation. *Appl Environ Microbiol* **64**, 1526–1531.
- Inaoka, T., Matsumura, Y. & Tsuchido, T. (1999). SodA and manganese are essential for resistance to oxidative stress in growing and sporulating cells of *Bacillus subtilis*. *J Bacteriol* **181**, 1939–1943.
- Keren, I., Kaldalu, N., Spoering, A., Wang, Y. & Lewis, K. (2004a). Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**, 13–18.
- Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. (2004b). Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186**, 8172–8180.
- Korch, S. B., Henderson, T. A. & Hill, T. M. (2003). Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol Microbiol* **50**, 1199–1213.
- Lewis, K. (2001). Riddle of biofilm resistance. *Antimicrob Agents Chemother* **45**, 999–1007.
- Lewis, K. (2005). Persister cells and the riddle of biofilm survival. *Biochemistry* **70**, 267–274.
- Lloyd-Jones, G., Osborn, M., Ritchie, D. A., Strike, P., Hobman, J. L., Brown, N. L. & Rouch, D. A. (1994). Accumulation and intracellular fate of tellurite in tellurite-resistant *Escherichia coli*: a model for the mechanism of resistance. *FEMS Microbiol Lett* **118**, 113–119.
- Lohmeier-Vogel, E. M., Ung, S. & Turner, R. J. (2004). In vivo ^{31}P nuclear magnetic resonance investigation of tellurite toxicity in *Escherichia coli*. *Appl Environ Microbiol* **70**, 7342–7347.
- Mages, M., Óvári, M., Tümpling, W., Jr & Kröpfl, K. (2004). Biofilms as bio-indicator for polluted waters? Total reflection X-ray fluorescence analysis of biofilms of the Tisza River (Hungary). *Anal Bioanal Chem* **378**, 1095–1101.
- Moran, L. A., Scrimgeour, K. G., Horton, H. R., Ochs, R. S. & Rawn, J. D. (1994). Glycolysis. In *Biochemistry*, 2nd edn. Upper Saddle River, NJ: Prentice Hall.
- Morck, D. W., Lam, K., McKay, S. G., Olson, M. E., Prosser, B., Ellis, B. D., Cleeland, R. & Costerton, J. W. (1994). Comparative evaluation of fleroxacin, ampicillin, trimethoprim-sulfamethoxazole, and gentamicin as treatments of catheter-associated urinary tract infection in a rabbit model. *Int J Antimicrob Agents* **4**, S21–S27.
- Moyed, H. S. & Bertrand, K. P. (1983). *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol* **155**, 768–775.
- Mukhopadhyay, R., Rosen, B. P., Phung, L. T. & Silver, S. (2002). Microbial arsenic: from geocycles to genes and enzymes. *FEMS Microbiol Rev* **26**, 311–325.
- Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G. & Read, R. R. (2002). Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* **66**, 86–92.
- Oremland, R. S. & Stolz, J. F. (2003). The ecology of arsenic. *Science* **300**, 939–944.
- Potrykus, J. & Węgrzyn, G. (2004). The *ypdI* gene codes for a putative lipoprotein involved in the synthesis of colanic acid in *Escherichia coli*. *FEMS Microbiol Lett* **235**, 265–271.
- Scherrer, R. & Moyed, H. S. (1988). Conditional impairment of cell division and altered lethality in *hipA* mutants of *Escherichia coli* K-12. *J Bacteriol* **170**, 3321–3326.
- Schützendübel, A. & Polle, A. (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *J Exp Bot* **53**, 1351–1365.
- Spoering, A. L. & Lewis, K. (2001). Biofilm and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**, 6746–6751.
- Stanley, N. R. & Lazazzera, B. A. (2004). Environmental signals and regulatory pathways that influence biofilm formation. *Mol Microbiol* **52**, 917–924.
- Stewart, P. S. (2002). Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* **292**, 107–113.
- Stewart, P. S. (2003). Diffusion in biofilms. *J Bacteriol* **185**, 1485–1491.
- Stewart, P. S., Rayner, J., Roe, F. & Rees, W. M. (2001). Biofilm penetration and disinfection efficacy of alkaline hypochlorite and chlorosulfamates. *J Appl Microbiol* **91**, 525–532.
- Stohs, S. J. & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of metal ions. *Free Radic Biol Med* **18**, 321–336.
- Sutherland, I. W. (2001). Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology* **147**, 3–9.
- Teitzel, G. M. & Parsek, M. R. (2003). Heavy metal resistance of biofilm and planktonic *Pseudomonas aeruginosa*. *Appl Environ Microbiol* **69**, 2313–2320.

- Trutko, S. M., Akimenko, V. K., Suzina, N. E., Anisimova, L. A., Shlyapnikov, M. G., Baskunov, B. P., Duda, V. I. & Boronin, A. M. (2000). Involvement of the respiratory chain of Gram-negative bacteria in the reduction of tellurite. *Arch Microbiol* **173**, 178–186.
- Turner, R. J. (2001). Tellurite toxicity and resistance in Gram-negative bacteria. *Recent Res Dev Microbiol* **5**, 69–77.
- Turner, R. J., Weiner, J. H. & Taylor, D. E. (1992). Use of diethyldithiocarbamate for quantitative determination of tellurite uptake by bacteria. *Anal Biochem* **204**, 292–295.
- Turner, R. J., Weiner, J. H. & Taylor, D. E. (1998). Selenium metabolism in *Escherichia coli*. *BioMetals* **11**, 223–227.
- Turner, R. J., Weiner, J. H. & Taylor, D. E. (1999). Tellurite-mediated thiol oxidation in *Escherichia coli*. *Microbiology* **145**, 2549–2557.
- Turner, R. J., Aharonowitz, Y., Weiner, J. H. & Taylor, D. E. (2001). Glutathione is a target in tellurite toxicity and is protected by tellurite resistance determinants in *Escherichia coli*. *Can J Microbiol* **47**, 33–40.
- Walters, M. C., III, Roe, F., Bugnicourt, A., Franklin, M. J. & Stewart, P. S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**, 317–323.
- Whitfield, C. & Roberts, I. S. (1999). Structure, assembly and regulation of expression of capsules in *Escherichia coli*. *Mol Microbiol* **31**, 1307–1319.
- Xu, K. D., McFeters, G. A. & Stewart, P. S. (2000). Biofilm resistance to antimicrobial agents. *Microbiology* **146**, 547–549.