Microbiology (2010), **156**, 30–38 DOI 10.1099/mic.0.029751-0

Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of *Pseudomonas aeruginosa* grown in continuous culture

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Received 2 April 2009
Revised 5 October 2009
Accepted 6 October 2009

This study investigates the link between adaptation to biocides and antibiotics in *Pseudomonas aeruginosa*. An enrichment continuous culture of *P. aeruginosa* NCIMB 10421 (MIC 25 mg BKC l^{-1}) was operated (D=0.04 h^{-1}, 792 h) with added benzalkonium chloride (BKC). A derivative, PA-29 (696 h), demonstrated a >12-fold decrease in sensitivity to the biocide (MIC >350 mg BKC l^{-1}). The variant demonstrated a 256-fold increase in resistance to ciprofloxacin, with a mutation in the *gyrA* gene (Thr-83→Ile). Similarly, culturing of the original strain in a continuous-culture system with ciprofloxacin selection pressure led to the evolution of BKC-adapted populations (MIC 100 mg BKC l^{-1}). Efflux pump activity predominantly contributed to the developed phenotype of PA-29. An amino acid substitution (Val-51→Ala) in *nfxB*, the Mex efflux system regulator gene, was observed for PA-29. Overexpression of both MexAB-OprM and MexCD-OprJ was recorded for PA-29. Similarly, *mexR*, a repressor of the Mex system, was downregulated. Competition studies were carried out in continuous culture between PA-29 and the original strain (in the presence of subinhibitory concentrations of BKC). The outcome of competition was influenced by the concentration of biocide used and the nature of limiting nutrient. The inclusion of 1 mg BKC l^{-1} in the medium feed was sufficient to select (S=0.011) for the BKC-adapted strain in magnesium-limited culture. Conversely, the presence of 10 mg BKC l^{-1} in the medium supply was insufficient to select for the same organism (S=−0.017) in the glucose-limited culture. These results indicate the importance of environmental conditions on selection and maintenance of biocide adaptation.

INTRODUCTION

Bacteria are constantly challenged by a gradient of biocide concentration from a subinhibitory level up to an inhibitory level in domestic, health-care and industrial environments. There is increasing concern that the use of biocides [such as benzalkonium chloride (BKC) and other quaternary ammonium compounds] in these environments may be contributing to the development of micro-organisms with decreased susceptibilities to antibiotics and disinfectants (Rutala *et al.*, 1997; Russell *et al.*, 1998; Russell, 1999, 2002; McBain & Gilbert, 2001; Fraise, 2002; Langsrud *et al.*, 2003; Thorrold *et al.*, 2007).

There is some evidence to suggest that micro-organisms that have become less sensitive to disinfectants (because of exposure to biocides) also show decreased susceptibilities to some antibiotics (Irizarry *et al.*, 1996; Moken *et al.*, 1997; Akimitsu *et al.*, 1999; Noguchi *et al.*, 1999; Price *et al.*, 2002; McMahon *et al.*, 2007; Karatzas *et al.*, 2007). Others indicate that bacterial co-adaptation to biocides and antibiotics is rare and has little clinical significance (Stecchini *et al.*, 1992; Fernandez-Astorga *et al.*, 1995; Anderson *et al.*, 1997; To *et al.*, 2002; Cole *et al.*, 2003).

Benzalkonium chloride (BKC: alkyl dimethyl benzyl ammonium chloride) is a nitrogen-based quaternary ammonium compound demonstrating broad-spectrum antimicrobial activity. Low-level and greater magnitude of adaptation to the biocide has been noted in strains of *Pseudomonas aeruginosa* (Langsrud *et al.*, 2003). Decreased susceptibility to biocides in *P. aeruginosa* is thought to be mediated primarily by the action of efflux pumps (Piddock, 2006; Chuanchuen *et al.*, 2001), most notably the Mex systems (Lomovskaya *et al.*, 1999).

A principal aim of this study was to determine if subjecting populations of *P. aeruginosa* NCIMB 10421 to increasing levels of BKC selection pressure in long-term continuous

Abbreviations: BKC, benzalkonium chloride; EPI, efflux pump inhibitor; MSC minimal selection concentration; QRDR, quinolone-resistance-determining region; Rti-PCR, real-time PCR.

culture would result in cross-adaptation to antimicrobials. We have previously shown that subjecting populations of *Escherichia coli* to levofloxacin selection pressure in chemostats will result in the evolution of populations showing increased resistance to levofloxacin (Fleming *et al.*, 2002). Furthermore, in that study, we identified the resulting minimal selection concentration (MSC), the lowest concentration of the drug that gave the resistant strain a selective advantage when competed against the sensitive phenotype (O'Reilly & Smith, 1999). This study investigated whether the source of a genuine nutrient limitation in chemostat culture might influence the MSC in the absence and presence of subclinical levels of biocide.

METHODS

Micro-organisms and media. *Pseudomonas aeruginosa* NCIMB 10421 was the original strain used for this study. All other strains were variants of 10421, generated by means of selective continuous culture with BKC selection pressure. Developed strains were stored at −20 °C in 20 % (v/v) glycerol. Stocks were retrieved on Nutrient agar (Oxoid, 18 h of growth, 37 °C). M9 minimal medium (Maniatis, 1982) was adjusted to produce both glucose- and magnesium-limiting media for competition studies. The glucose concentration was reduced from the recommended 2 g l^{-1} to 1.2 g l^{-1}, producing a glucose-limited medium. The magnesium sulfate concentration was reduced from the recommended 2 mM to 1.2 mM concentrations to produce a magnesium-limited medium. Genuine nutrient limitation (glucose or magnesium) was assured using the Goldberg & Er-el (1981) method. BKC (Sigma-Aldrich) served as a selective agent in continuous cultures and for antimicrobial susceptibility assays; it was diluted to appropriate working concentrations using sterile deionized water.

Antimicrobial susceptibility testing. Minimum inhibitory concentrations (MICs) for BKC were determined by the macro-broth dilution method of NCCLS (M7-A6, NCCLS, 2003). The standard assay procedure was unaltered for derivatives of *P. aeruginosa* 10421. Antibiotic sensitivity was determined using Epsilometer strips (Biodisk). The MIC profile was determined for tobramycin (0.016–128 μg), gentamicin (0.016–128 μg), aztreonam (0.016–128 μg), ciprofloxacin (0.016–128 μg), imipenem (0.002–32 μg), polymyxin B (0.064–128 μg), amikacin (0.016–128 μg), vancomycin (0.016–128 μg) and minocycline (0.016–128 μg).

Maximum specific growth rates (μ_{max}) of original and chemostat-derived strains. μ_{max} determinations were carried out in Mueller–Hinton broth (MHB; Oxoid) medium using a multi-well plate reader (GENios Tecan, AG Trading). Aliquots (5 μl) of *P. aeruginosa* 10421 or its derivatives (10^6 c.f.u. ml^{-1}) were inoculated to 250 μl of appropriate medium contained in micro-titre plate wells. Optical density (OD_{595}) measurements were taken over the course of 24 h (37 °C). Growth rates were determined from plots of log OD_{595} versus time, by the method of Schwartz *et al.* (1988), and were the result of at least five replicates.

Enrichment for BKC-adapted variants of *P. aeruginosa* 10421, by means of selective chemostat culture. The enrichment chemostat apparatus used was described previously (Fleming & Patching, 2008). The culture was inoculated with *P. aeruginosa* 10421 previously grown overnight in 20 ml Nutrient broth (37 °C, 125 r.p.m.). The vessel was brought to its working volume (480 ± 10 ml quarter-strength Nutrient broth). The culture was allowed to proliferate (D=0.04 h^{-1}, 37 °C). Medium flow to the culture was controlled using a peristaltic pump (Watson-Marlow 505U). After six generations of growth, the medium supply was supplemented with 12.5 mg BKC l^{-1}. This represented 50 % of the MIC of *P. aeruginosa* 10421 for the biocide. Biocide concentrations were further increased when the OD_{540} of the culture recovered to ~70 % of the value recorded before addition of the biocide. Samples were withdrawn routinely, and stored in glycerol (20 %, v/v) at −20 °C for further MIC analysis.

Screening for variants with increased MICs for BKC. Derivatives of *P. aeruginosa* 10421 were deemed to be adapted to the biocide when MIC determinations demonstrated a threefold increase in broth MIC (BKC) compared with that of the progenitor strain. The MIC (antibiotics and biocide) stability of both *P. aeruginosa* 10421 and its derivatives was determined after serial transfer (24 h, 37 °C) of cultures in biocide-free medium for 20 days.

Competition studies between original and BKC-adapted strains in continuous culture. Quarter-strength Nutrient broth (Oxoid), or glucose/magnesium-limited M9 minimal medium was used for short-term competition studies. Individual continuous culture systems were established with *P. aeruginosa* 10421 and an enrichment-culture derivative. Cultures were allowed to reach steady state (D=0.2 h^{-1}, 20 h) before transfer to a third chemostat vessel. On commencement of sampling, the original strain constituted ~80 % of the total population. The medium feed without or containing the biocide (1, 5 or 10 mg BKC l^{-1}) was established immediately after mixing of cultures. Equal concentrations of BKC were added directly to the chemostat vessel. Samples (2 ml) were withdrawn at denoted intervals, and plated to Nutrient agar (24 h, 37 °C). Using the replica plate stick method, at least 100 discrete colonies were transferred to agar plates with and without 500 mg BKC l^{-1} supplementation. The relative proportions of competing strains were determined after incubation of the resultant plates (37 °C, 24 h). A selection coefficient (S, h^{-1}) was calculated using the equation of Dykhuizen (1993):

$$\ln[X_1(t)/X_2(t)]=\ln[X_1(0)/X_2(0)]-S(t)$$

where $X_1(t)$ and $X_2(t)$ represent the relative proportion of the two competing strains at time t (measured in h). S is a measure of differential growth rate per unit time (selection coefficient). Selection coefficients were expressed on a selection-per-hour basis. The strain designated by X_1 is favoured, neutral, or disfavoured relative to the strain designated by X_2 as $S>0$, $S=0$, or $S<0$.

Effect of efflux pump inhibitor on antimicrobial sensitivity. Phe-Arg β-naphthylamide dihydrochloride (Sigma Aldrich) is an efflux pump inhibitor (EPI). The presence of an EPI reduces the susceptibility of a strain displaying efflux-mediated adaptation to an antimicrobial agent (Lomovskaya & Bostian, 2006). MICs of *P. aeruginosa* 10421 and its enrichment-culture derivative were determined in the presence and absence of 40 mg EPI l^{-1}.

PCR amplification and sequencing of *gyrA*, *mexR*, *nfxB* and *parC*. Target regions of *P. aeruginosa* PA-29 were amplified using specific primers (Table 1), purchased from Eurofins MWG. PCR was carried out using Phusion, a high-fidelity DNA polymerase (Finnzymes Diagnostics). Reactions were carried out in a 50 μl final volume with 1 μl heat-extracted template DNA, 0.5 μl Phusion DNA polymerase and 0.5 μM of each primer. Reactions were performed in a Mastercycler Gradient (Eppendorf), for 35 cycles. Initial denaturation was for 30 s at 98 °C, cycle denaturation was 10 s, annealing for 30 s at 68 °C (*gyrA*), 63 °C (*parC*), 61 °C (*mexR*), or 66 °C (*nfxB*). Extension was for 30 s at 72 °C. PCR products were examined on 0.75 % agarose gels. PCR products were eluted from the gel using the QIAquick-spin PCR purification kit (Qiagen). Purified PCR-amplified DNA was sequenced by GATC Biotech. Comparison of

Table 1. Primers used for sequencing and RTi-PCR

Primer	Nucleotide sequence (5′ to 3′)	Use	Reference
GyrA_for	GACGGCCTGAAGCCGGTGCAC	Sequencing	Lee *et al.* (2005)
GyrA_rev	GCCCACGGCGATACCGCTGGA		
ParC_for	CGAGCAGGCCTATCTGAACTAT	Sequencing	Lee *et al.* (2005)
ParC_rev	GAAGGACTTGGGATCGTCCGGA		
MexR_for	TCGGCCAAACCAATGAACTAC	Sequencing	Jalal & Wretlind (1998)
MexR_rev	GGGTGAGCGGGGCAAACAACT		
NfxB_for	CGCCCCGATCCTTCCTATTGC	Sequencing	Jalal & Wretlind (1998)
NfxB_rev	ACGAGCGTCACGGTCCTTTGC		
MexB_for	TTGATAGGCCCATTTTCGCGT	RTi-PCR	This study
MexB_rev	TCTGCTGCTCGATCACCTGGA		
MexD_for	CGAATTCTTCATCAAGCGGCC	RTi-PCR	This study
MexD_rev	AGCACACTGGTGACGGAGTCCA		
MexF_for	CTCCCAATTCTTCATCCAGCGG	RTi-PCR	This study
MexF_rev	GATGACTTTCGGGTTGGCGC		
MexY_for	GACCACCAGGAAGAACAGCGGT	RTi-PCR	This study
MexY_rev	TCATGACCTCGCTGGCGTTC		
ProC_for	CCTGCTCCACCAGTGCTT	RTi-PCR	This study
ProC_rev	CTGTCCAGCGAGGTCGAG		

experimentally determined nucleotide sequences against sequence databases was performed with the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene expression analysis of *mexB*, *mexD*, *mexF*, *mexY* and *mexR* by real-time PCR. RNA was extracted and purified using the SV Total RNA Isolation kit (Promega) from *P. aeruginosa* 10421 grown to exponential phase (OD_{600} 0.6) in MHB (37 °C), and PA-29 grown to exponential phase in the presence and absence of 150 mg BKC l^{-1}.

Total RNA was quantified using a NanoDrop 3300 (Thermo Scientific) and stored at −70 °C until used in cDNA preparation. Total RNA (1 μg) was used as a template in reverse transcriptase PCR. Reverse transcriptase PCR was carried out in a 20 μl volume, using the QuantiTect reverse transcription kit (Qiagen). Primers are listed in Table 1. Resulting cDNA was quantified using a NanoDrop 3300 and stored at −20 °C until use in real-time PCR (RTi-PCR). RTi-PCR was carried out in a final volume of 10 μl, containing 2.5 μl cDNA (100 ng cDNA μl^{-1}), 1.5 μl H_2O, 0.5 μM of each primer, and 5 μl of 2× QuantiTect SYBR Green PCR Master Mix (Qiagen). RTi-PCR was performed in a LightCycler 480 (Roche). The following temperature profile was used: an initial denaturation for 15 min at 95 °C, followed by 45 cycles including denaturation for 15 s at 94 °C, annealing for 30 s at 56 °C, and an extension for 30 s at 72 °C. The *proC* gene, encoding a pyrroline-5-carboxylate reductase, was used as a reference gene for normalizing the transcription levels of target genes. This gene was previously shown to be a suitable reference gene for RTi-PCR (Savli *et al.*, 2003). The mean expression for the three different samples of cDNA was calculated according the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). Assays were performed (n=6) from three biological replicates.

RESULTS AND DISCUSSION

Original strain characteristics and enrichment study

P. aeruginosa 10421 (original strain) had an MIC of 25 mg BKC l^{-1}. In order to select for spontaneous variants with elevated MICs for BKC, 10421 was grown in a continuous culture (D=0.04 h^{-1}, 792 h, 33 generations), using quarter-strength Nutrient broth. Concentrations of BKC in the medium feed were increased at two-generation intervals when OD_{595} and total viable counts returned to 70 % of that recorded at the previous addition (Fig. 1). The MIC for BKC of the chemostat population increased 12-fold between the start and termination of the culture at 792 h (Fig. 1). This degree of adaptation to biocide in continuous culture system is some fourfold greater than that observed for strains adapted using a simple serial batch culture (Tabata *et al.*, 2003). Furthermore, in this study, developed strains showed stable inheritance of the characteristic when cultured in the absence of BKC selection pressure. Simple batch-culture-based subculturing techniques often result in unstable adapted phenotypes, in both *P. aeruginosa* and other species (Jones *et al.*, 1989; Méchin *et al.*, 1999; Suller & Russell, 2000). Eight strains that had elevated MICs for BKC were isolated throughout the course of the enrichment continuous culture (Table 2). Four of these strains showed an MIC of >200 mg BKC l^{-1}, an eightfold decreased susceptibility compared with the original strain. These strains were, however, growth rate disadvantaged compared to the original strain (Table 2). One isolate, which was retained for further study (PA-29), showed a μ_{max} similar to that of the original strain.

The antibiotic susceptibility profile for a number of antimicrobials was also determined for the continuous-culture isolates (Table 2). This combination of antibiotics was chosen so as to reflect the range of targets at which biocides are known to act. Significant changes in susceptibility to minocycline and ciprofloxacin were noted. *P. aeruginosa* 10421 was phenotypically resistant to minocycline (MIC >128 mg l^{-1}). Derivatives isolated after 25 generations of growth in the chemostat vessel were more sensitive to minocycline (MIC 16–48 mg l^{-1}). An increase in sensitivity

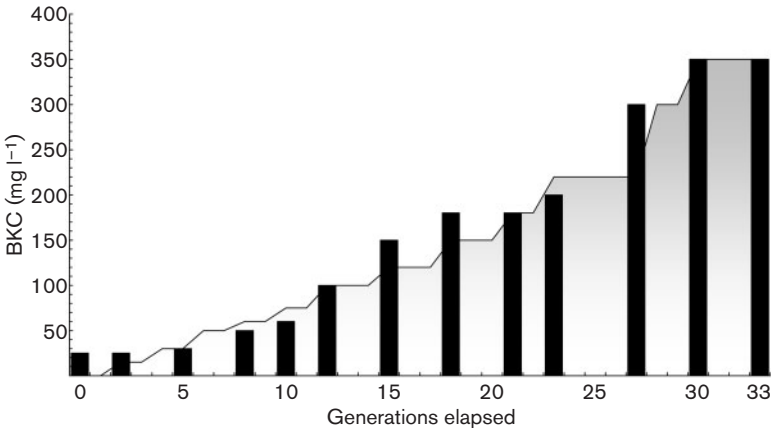

Fig. 1. Enrichment culture of *P. aeruginosa* 10421 in complex medium (D=0.04 h^{-1}). BKC added to the medium reservoir is represented by the shaded area. MICs for BKC (mg l^{-1}) for continuous culture populations tested are represented by the solid bars.

to polymyxin B was noted, although it was not of the same magnitude, and may be of little clinical significance. Isolates also demonstrated a decreased susceptibility for ciprofloxacin (Table 2). *P. aeruginosa* 10421 had an MIC of 0.125 mg ciprofloxacin l^{-1}. PA-29 and PA-33 demonstrated an MIC of 32 mg ciprofloxacin l^{-1}. Thus it would appear that the application of BKC selection pressure to a chemostat with *P. aeruginosa* 10241 resulted in the evolution of variants showing resistance to ciprofloxacin. A further enrichment culture was operated with *P. aeruginosa* 10241 (624 h, D=0.04 h^{-1}) in which BKC selection pressure was replaced by ciprofloxacin selection pressure (not shown). The degree of selection pressure applied was 0.25 MIC for ciprofloxacin at the start of the culture and this was increased incrementally to 40 mg ciprofloxacin l^{-1} over 576 h. Populations were examined afterwards for their sensitivity to BKC. Variants evolved in the reactor which demonstrated a significant adaptation to ciprofloxacin (MIC >128 mg l^{-1}) and BKC (MIC 100 mg l^{-1}). Ciprofloxacin-selected populations did not display significant changes in sensitivity to antibiotics other than those of the quinolone group (levofloxacin, nalidixic acid). It is thus evident that the application of ciprofloxacin selection pressure to the culture resulted in a co-adaptation to BKC. Previous work by Loughlin *et al.* (2002) obtained BKC-adapted populations of *P. aeruginosa* using traditional serial batch culture methods. The resulting strains did not show resistance to clinically relevant antibiotics, including ciprofloxacin, although they did show resistance to other quaternary ammonium compounds. The different results from the present study may indicate the importance of growth conditions in the specific response of *P. aeruginosa* to BKC adaptation/selection.

Sequence analysis of the QRDR region of *gyrA* and *parC*

The quinolone-resistance-determining region (QRDR) of *gyrA* and *parC* was examined in the PA-29 strain. Sequence

Table 2. Characteristics (μ_{max}, MIC for BKC and MIC for antibiotics: E-test) of chemostat enrichment-derived variants

The μ_{max} values shown were determined in Mueller–Hinton medium (n=5). All MIC values are the mean of three determinations. Antibiotics: MC, minocycline; TOB, tobramycin; AZ, aztreonam; CIP, ciprofloxacin; PB, polymyxin B; AK, amakacin; GE, gentamicin; VA, vancomycin; IP, imipenem.

Isolate*	μ_{max} (h^{-1})	MIC (mg BKC l^{-1})	MIC using E-test methodology (mg l^{-1})								
			MC	TOB	AZ	CIP	PB	AK	GE	VA	IP
10421	1.22±0.05	25	>128	1.5	3	0.125	4	8	4	>128	2
PA-3	0.78±0.1	40	>128	1.5	2	0.250	3	6	4	>128	1.5
PA-7	0.99±0.07	50	96	1.5	3	2	3	6	4	>128	2
PA-11	1.01±0.08	80	96	1	3	2	1.5	6	6	>128	1.5
PA-19	1.06±0.07	180	96	1.5	2	8	1.5	4	4	>128	0.75
PA-23	0.93±0.08	200	96	1.5	3	8	3	8	6	>128	0.75
PA-27	1.11±0.06	300	48	1	2	16	3	6	4	>128	2
PA-29	1.18±0.05	>350	48	1.5	2	32	1.5	8	6	>128	1.5
PA-33	0.99±0.08	>350	16	1	3	32	2	6	6	>128	2

*The number after the hyphen in the PA isolates indicates the number of generations elapsed of culture enrichment.

analysis of *parC* yielded only a silent mutation. PA-29 possessed a Thr-83→Ile substitution in GyrA. Ciprofloxacin-resistant *P. aeruginosa* were previously described by Higgins *et al.* (2003) carrying the same substitution. Indeed, the study concluded that mutations in the Thr-83 codon were the primary cause of reduced fluoroquinolone sensitivity in *P. aeruginosa*.

Expression of efflux pumps and their possible role in decreased sensitivity to BKC and ciprofloxacin

The MexAB-OprM, MexCD-OprJ, MexEF-OprN and MexXY-OprM systems of *P. aeruginosa* possess overlapping spectra (Lomovskaya *et al.*, 1999). They can extrude a range of antibacterial compounds. Resistance to fluoroquinolones has been shown to be mediated in *P. aeruginosa* by efflux pump systems (Poole, 2005; Thorrold *et al.*, 2007).

In the present study, sequence analysis of *mexR*, a repressor of the MexAB-OprM efflux system, in PA-29 did not reveal any mutations. A point mutation was identified in the *nfxB* gene, a regulator of the Mex system, of PA-29. This point mutation led to an amino acid substitution (Val-51→Ala). Amino acid substitutions throughout the *nfxB* gene were previously reported (Higgins *et al.*, 2003; Jalal & Wretlind, 1998), though not at this codon.

Changes in expression of the *mexB*, *mexD*, *mexF* and *mexY* genes of PA-29 were measured under two growth conditions, in the absence and presence of 150 mg BKC l^{-1} (between 5 and 6 MIC of the original strain). Expression was compared to that of 10421. Addition of BKC to the growth medium (at a level inhibitory to the original strain) ensured the expression of the BKC-adapted phenotype. As evident in Fig. 2, expression of both *mexB* (32-fold) and *mexD* (14-fold) had increased in the BKC-adapted PA-29. Increased expression was augmented by the addition of BKC to the growth medium (respective 125- and 102-fold increases were recorded). Expression of *mexF* and *mexY* in PA-29 was half that of 10421 in the absence of BKC, although expression of *mexY* was greater than 10421 (1.7-fold) in the presence of BKC. The level of expression of *mexR*, a repressor of the MexAB-OprM system, decreased greatly in PA-29. A 28-fold decrease in expression was noted in the absence of BKC, and an 88-fold decrease in expression in the presence of BKC.

The degree to which these efflux pumps confer antimicrobial insusceptibility to PA-29 was determined. MICs were determined in the absence and presence of an efflux pump inhibitor (EPI) previously shown to be effective against the aforementioned pumps (Lomovskaya *et al.*, 1999). These experiments showed that efflux systems in isolate PA-29 play a role in developed insusceptibility toward both BKC and ciprofloxacin. The susceptibility of the isolate to BKC increased in the presence of the EPI (>350 mg BKC l^{-1} to 50 mg BKC l^{-1}). Similar patterns were recorded in response to ciprofloxacin. PA-29 exhibited a decrease in the MIC of this antibiotic in the presence of the EPI (32 mg ciprofloxacin l^{-1} to 6 mg ciprofloxacin l^{-1}). A triclosan-adapted *P. aeruginosa* mutant hyper-expressing MexCD-OprJ was previously described by Chuanchuen *et al.* (2001). The mutant also demonstrated a 94-fold decrease in sensitivity to ciprofloxacin.

An attempt was made to determine the susceptibility of PA-29 and the original strain to novobiocin and oxacillin, other antimicrobials subject to efflux. 10421 and PA-29 were clinically resistant to both (MICs >2048 mg l^{-1}) in the absence of the EPI. PA-29 and 10421 displayed increased sensitivity to novobiocin in the presence of the EPI, with respective MICs of 256 and 128 mg novobiocin l^{-1}, again indicative of the increased efflux activity of PA-29. Sensitivity to oxacillin in the presence of the EPI could not be determined, due to the precipitation of the compounds at increased concentrations.

Previously, adaptation to the biocide triclosan was shown to be wholly a result of increased efflux in *P. aeruginosa* (Chuanchuen *et al.*, 2003). It is evident that BKC adaptation and the co-resistance to ciprofloxacin of strain PA-29 are predominantly mediated by the action of the Mex efflux system. However, the susceptibility of PA-29 to the biocide and antibiotic in the presence of the EPI did

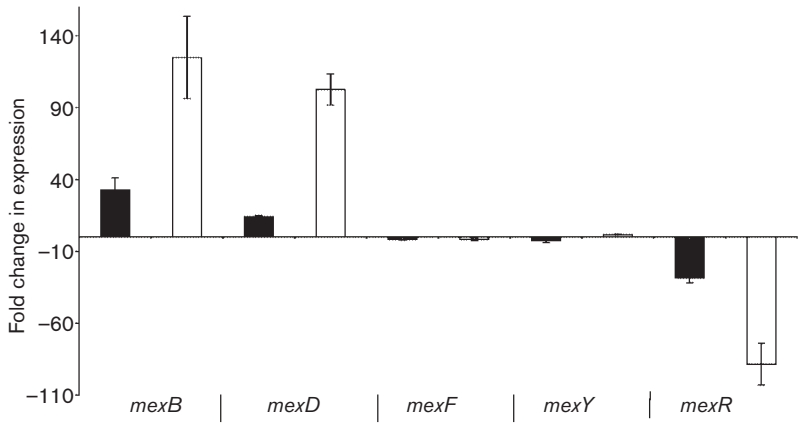

Fig. 2. Fold changes in expression of *mexB*, *mexD*, *mexF*, *mexY* and *mexR* in PA-29 cultured in the absence (black bars) and presence (white bars) of 150 mg BKC l^{-1} compared with 10421. The values plotted are the result of RTi-PCR performed on six replicates from three biological replicates (means ± SD).

not increase to the baseline levels demonstrated by the original strain. This may suggest that further mechanisms (or other efflux pumps), in conjunction with the mutation in the QRDR, are also responsible for the adapted phenotype of PA-29. Conversely, the EPI may not fully inhibit efflux activity of the Mex system as it competes with the substrate. Results from the present study would indicate that the MexXY-OprM system was not involved in the adaptation to BKC. Resistance to tigecycline, an analogue of minocycline, was previously shown to be mediated by the MexXY-OprM efflux pump (Dean *et al.*, 2003). Decreased expression of the system may explain the increased sensitivity of PA-29 to minocycline.

Chemostat competition studies and determination of the MSC

The BKC-adapted strain PA-29 was competed against *P. aeruginosa* 10421 in continuous culture. Competition studies were performed under a variety of nutrient limitations for 30 h in the absence and presence of BKC concentrations deemed subinhibitory to the original strain (1, 5 and 10 mg BKC l^{-1}). The concentrations used were insufficient to select for BKC-adapted variants in a homogeneous 10421 population within the same time frame. Competition between the adapted and original strain in the absence of BKC supplementation resulted in the dominance of the original strain irrespective of nutrient limitation (Table 3). The magnitude of the dominance (as described by the selection coefficients, *S*, derived from the slopes of Fig. 3a–c) was dependent on the nature of the limiting nutrient (glucose, magnesium or unknown: Table 3). This would suggest a metabolic burden associated with adaptation to BKC. Glucose limitation represents a low-energy environment, wherein the disadvantage of a metabolic burden would be an important factor in outcome. Magnesium is integral to the structure of LPS, the outermost component of the cell, previously shown to be linked with susceptibility to disinfectants (Manzoor *et al.*, 1999). EDTA is often used in conjunction with disinfectant as a chelating agent for available magnesium, accentuating the effects of the biocide.

Table 3. Selection coefficients (*S*) derived from continuous-culture competition studies between *P. aeruginosa* 10421 and PA-29

Studies were performed in complex medium or a glucose-limited or magnesium-limited medium. Positive *S* values denote competition in favour of the continuous culture derivative. Values are derived from the slopes of graphs in Fig. 3(a–c).

	Medium	BKC (mg l^{-1})	*S* (h^{-1})
PA-29 vs10421	Nutrient broth	0	−0.027
		1	−0.001
		5	0.039
		10	0.113
PA-29 vs10421	Glucose-limiting M9 medium	0	−0.066
		1	−0.044
		5	−0.039
		10	−0.017
PA-29 vs10421	Magnesium-limiting M9 medium	0	−0.015
		1	0.011
		5	0.055
		10	0.123

Supplementation of BKC to the medium flow altered the selection process. Concentrations of 5 and 10 mg BKC l^{-1} were sufficient to select for the adapted organism in Nutrient broth (Table 3). Concentrations of 1, 5 and 10 mg BKC l^{-1} were sufficient to select for the adapted organism when examined under magnesium limitation (Table 3). However, these same concentrations of BKC were insufficient to select for BKC-adapted PA-29 in a mixed culture under glucose limitation (Table 2).

MSCs were calculated using the selection coefficients. The MSC for PA-29 in complex medium lay between 1 mg BKC l^{-1} and 5 mg BKC l^{-1}. This represented a challenge of between 4 and 20 % of the MIC of 10421. In the glucose-limiting environment, BKC concentrations tested were insufficient to confer a selective advantage to PA-29. The MSC for PA-29 in complex medium was determined to be between 0 and 1 mg BKC l^{-1}. This represented a challenge of between 0 and 5 % of the MIC of the original strain.

It is evident from this study that the presence of subinhibitory concentrations of BKC can allow selection of the adapted variant in a mixed population. Likewise, the selection process is dependent on two factors: the concentration of the biocide in the medium, and the nature of the limiting nutrient. Magnesium limitation would appear to be advantageous to a BKC-adapted variant of *P. aeruginosa*, whereas the low-energy environment of glucose-limited medium is disadvantageous. An important consideration is that subinhibitory concentrations of a biocide promoted the selection of a biocide-adapted, and critically, a ciprofloxacin-resistant strain of *P. aeruginosa*. Published MSC data (Fleming *et al.*, 2002; O'Reilly & Smith, 1999) are based on antibiotic resistance and selection. It would appear that the MSC is also an important parameter in discussing biocides.

Conclusions

It is clear from the present study that the presence of subinhibitory concentrations of BKC is sufficient to select for adapted variants of *P. aeruginosa* in a sensitive culture. Gilbert & McBain (2003) stated that 'in any environment there is likely to be a continuum of biocide concentration ranging from treatment concentration to nil'. This, in effect, presents subinhibitory concentrations in the

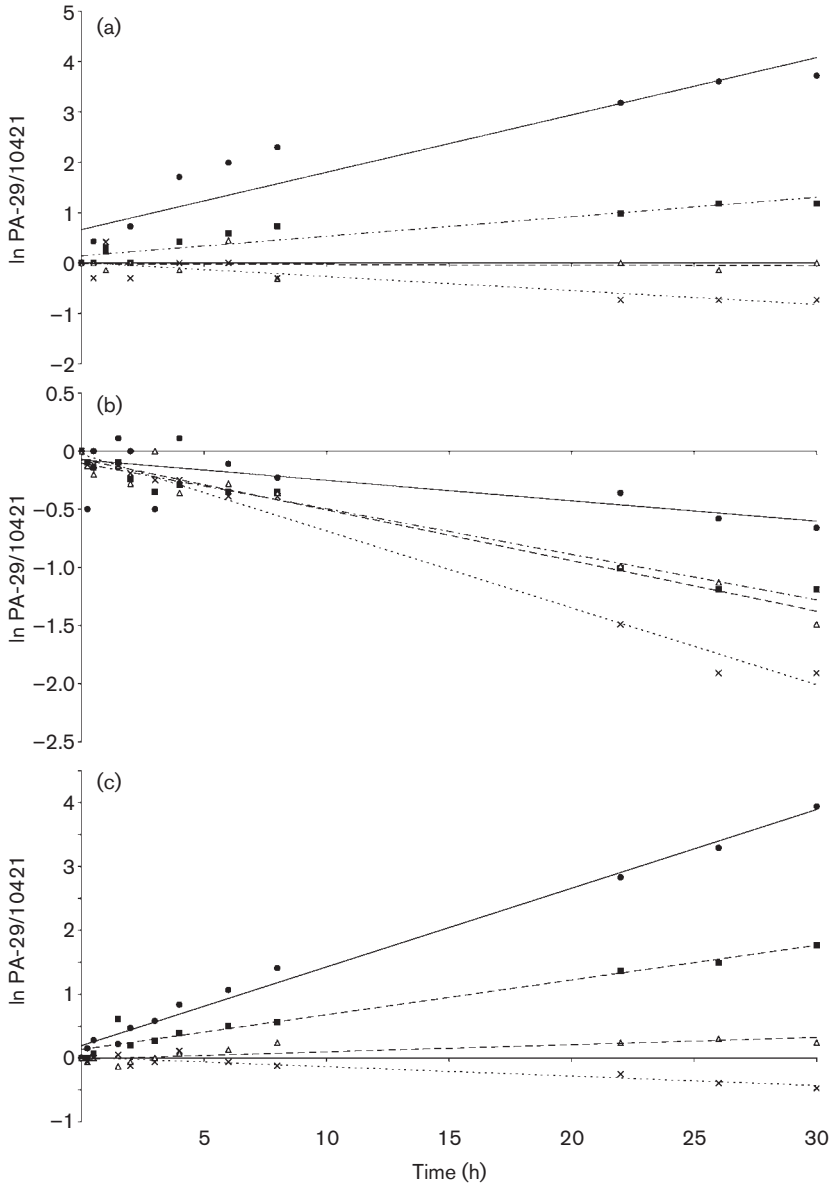

Fig. 3. Competition between 10421 and continuous culture isolate PA-29 in nutrient broth (a), in glucose-limited medium (b), and in magnesium-limited medium (c). Competition was carried out in cultures at $D=0.2\ h^{-1}$ in the absence (×) and presence of 1 (△), 5 (■) and 10 (●) mg BKC l^{-1}.

environment to which step-wise decreases in susceptibility may occur. The present study has examined the theory that adaptation to biocides can give rise to antibiotic resistance. This would seem to be dependent on the nature of the antibiotic and most likely the biocide and organism. BKC supplementation of the medium selected for a mutation in the QRDR of *P. aeruginosa*, thus selecting for fluoroquinolone resistance. Further to this, subinhibitory concentrations of biocide can actively maintain and select these co-adapted organisms in the natural environment. The concentration of the biocide and the nature of limiting nutrient play a significant role in the outcome of the selection process. Results of further studies based on examination of the theory would enhance our understanding of how micro-organisms adapt to antimicrobial agents and survive/proliferate in the environment. Studies of the adaptation process would benefit from a greater attention to the role of limiting nutrient and growth rate in the outcome and selection of adapted variants.

ACKNOWLEDGEMENTS

This research was supported by the Science Foundation of Ireland (SFI) (Basic Science award 04/BRT/BO446). P. H. Mc C. was supported by the Irish Research Council for Science, Engineering and Technology (IRCSET) under the Embark fellowship. A. A. O.-S. was supported under a Marie Curie Fellowship.

REFERENCES

Akimitsu, N., Hamamoto, H., Inoue, R. I., Shoji, M., Takemori, K., Hamasaki, N. & Sekimizu, K. (1999). Increase in resistance of methicillin-resistant *Staphylococcus aureus* to β-lactams caused by mutations conferring resistance to benzalkonium chloride, a dis-

infectant widely used in hospitals. *Antimicrob Agents Chemother* **43**, 3042–3043.

Anderson, R. L., Carr, J. H., Bond, W. W. & Favero, M. S. (1997). Susceptibility of vancomycin-resistant enterococci to environmental disinfectants. *Infect Control Hosp Epidemiol* **18**, 195–199.

Chuanchuen, R., Beinlich, K., Hoang, T. T., Becher, A., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2001). Cross-resistance between triclosan and antibiotics in *Pseudomonas aeruginosa* is mediated by multidrug efflux pumps: exposure of a susceptible mutant strain to triclosan selects *nfxB* mutants overexpressing MexCD-OprJ. *Antimicrob Agents Chemother* **45**, 428–432.

Chuanchuen, R., Karkhoff-Schweizer, R. R. & Schweizer, H. P. (2003). High-level triclosan resistance in *Pseudomonas aeruginosa* is solely a result of efflux. *Am J Infect Control* **31**, 124–127.

Cole, E. C., Addison, R. M., Rubino, J. R., Leese, K. E., Dulaney, P. D., Newell, M. S., Wilkins, J., Gaber, D. J., Wineinger, T. & Criger, D. A. (2003). Investigation of antibiotic and antibacterial agent cross-resistance in target bacteria from homes of antibacterial product users and nonusers. *J Appl Microbiol* **95**, 664–676.

Dean, C. R., Visalli, M. A., Projan, S. J., Sum, P. E. & Bradford, P. A. (2003). Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. *Antimicrob Agents Chemother* **47**, 972–978.

Dykhuizen, D. E. (1993). Chemostats used for studying natural selection and adaptive evolution. *Methods Enzymol* **224**, 613–631.

Fernandez-Astorga, A., Hijarrubia, M. J., Hernandez, M., Arana, I. & Sunen, E. (1995). Disinfectant tolerance and antibiotic resistance in psychrotrophic Gram-negative bacteria isolated from vegetables. *Lett Appl Microbiol* **20**, 308–311.

Fleming, G. & Patching, J. W. (2008). The fermenter in research and development. In *Practical Fermentation Technology*, pp. 347–377. Edited by B. Mc Neil & L. M. Harvey. Chichester: Wiley.

Fleming, G. T., McCarthy, D. M., Colombet, N. & Patching, J. W. (2002). The effect of levofloxacin concentration on the development and maintenance of antibiotic-resistant clones of *Escherichia coli* in chemostat culture. *J Ind Microbiol Biotechnol* **29**, 155–162.

Fraise, A. P. (2002). Biocide abuse and antimicrobial resistance – a cause for concern? *J Antimicrob Chemother* **49**, 11–12.

Gilbert, P. & McBain, A. J. (2003). Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance. *Clin Microbiol Rev* **16**, 189–208.

Goldberg, I. & Er-el, Z. (1981). The chemostat – an efficient technique for medium optimization. *Process Biochem* **16**, 2–4.

Higgins, P. G., Fluit, A. C., Milatovic, D., Verhoef, J. & Schmitz, F. J. (2003). Mutations in GyrA, ParC, MexR and NfxB in clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* **21**, 409–413.

Irizarry, L., Merlin, T., Rupp, J. & Griffith, J. (1996). Reduced susceptibility of methicillin-resistant *Staphylococcus aureus* to cetylpyridinium chloride and chlorhexidine. *Chemotherapy* **42**, 248–252.

Jalal, S. & Wretlind, B. (1998). Mechanisms of quinolone resistance in clinical strains of *Pseudomonas aeruginosa*. *Microb Drug Resist* **4**, 257–261.

Jones, M. V., Herd, T. M. & Christie, H. J. (1989). Resistance of *Pseudomonas aeruginosa* to amphoteric and quaternary ammonium biocides. *Microbios* **58**, 49–61.

Karatzas, K. A. G., Webber, M. A., Jorgensen, F., Woodward, M. J., Piddock, L. J. V. & Humphrey, T. J. (2007). Prolonged treatment of *Salmonella enterica* serovar Typhimurium with commercial disinfectants selects for multiple antibiotic resistance, increased efflux and reduced invasiveness. *J Antimicrob Chemother* **60**, 947–955.

Langsrud, S., Sundheim, G. & Borgmann-Strahsen, R. (2003). Intrinsic and acquired resistance to quaternary ammonium compounds in food-related *Pseudomonas* spp. *J Appl Microbiol* **95**, 874–882.

Lee, J. K., Lee, Y. S., Park, Y. K. & Kim, B. S. (2005). Alterations in the GyrA and GyrB subunits of topoisomerase II and the ParC and ParE subunits of topoisomerase IV in ciprofloxacin-resistant clinical isolates of *Pseudomonas aeruginosa*. *Int J Antimicrob Agents* **25**, 290–295.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_t}$ method. *Methods* **25**, 402–408.

Lomovskaya, O. & Bostian, K. A. (2006). Practical applications and feasibility of efflux pump inhibitors in the clinic – a vision for applied use. *Biochem Pharmacol* **71**, 910–918.

Lomovskaya, O., Lee, A., Hoshino, K., Ishida, H., Mistry, A., Warren, M. S., Boyer, E., Chamberland, S. & Lee, V. J. (1999). Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **43**, 1340–1346.

Loughlin, M. F., Jones, M. V. & Lambert, P. A. (2002). *Pseudomonas aeruginosa* cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics. *J Antimicrob Chemother* **49**, 631–639.

Maniatis, T. (1982). Appendix A. Biochemical techniques: liquid media. In *Molecular Cloning: a Laboratory Manual*, p. 440. Edited by T. Maniatis, E. F. Fritsch & J. Sambrook. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Manzoor, S. E., Lambert, P. A., Griffiths, P. A., Gill, M. J. & Fraise, A. P. (1999). Reduced glutaraldehyde susceptibility in *Mycobacterium chelonae* associated with altered cell wall polysaccharides. *J Antimicrob Chemother* **43**, 759–765.

McBain, A. J. & Gilbert, P. (2001). Biocide tolerance and the harbingers of doom. *Int Biodeterior Biodegradation* **47**, 55–61.

McMahon, M. A. S., Blair, I. S., Moore, J. E. & McDowell, D. A. (2007). Habituation to sub-lethal concentrations of tea tree oil (*Melaleuca alternifolia*) is associated with reduced susceptibility to antibiotics in human pathogens. *J Antimicrob Chemother* **59**, 125–127.

Méchin, L., Dubois-Brissonnet, F., Heyd, B. & Leveau, J. Y. (1999). Adaptation of *Pseudomonas aeruginosa* ATCC 15442 to didecyldimethylammonium bromide induces changes in membrane fatty acid composition and in resistance of cells. *J Appl Microbiol* **86**, 859–866.

Moken, M. C., McMurry, L. M. & Levy, S. B. (1997). Selection of multiple-antibiotic-resistant (*mar*) mutants of *Escherichia coli* by using the disinfectant pine oil: roles of the *mar* and *acrAB* loci. *Antimicrob Agents Chemother* **41**, 2770–2772.

NCCLS (2003). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. *Approved Standard M7–A6*. Wayne, PA: National Committee for Clinical Laboratory Standards.

Noguchi, N., Hase, M., Kitta, M., Sasatsu, M., Deguchi, K. & Kono, M. (1999). Antiseptic susceptibility and distribution of antiseptic-resistance genes in methicillin-resistant *Staphylococcus aureus*. *FEMS Microbiol Lett* **172**, 247–253.

O'Reilly, A. & Smith, P. (1999). Development of methods for predicting the minimum concentrations of oxytetracycline capable of exerting a selection for resistance to this agent. *Aquaculture* **180**, 1–11.

Piddock, L. J. V. (2006). Multidrug-resistance efflux pumps are not just for resistance. *Nat Rev Microbiol* **4**, 629–636.

Poole, K. (2005). Efflux-mediated antimicrobial resistance. *J Antimicrob Chemother* **56**, 20–51.

Price, C. T. D., Singh, V. K., Jayaswal, R. K., Wilkinson, B. J. & Gustafson, J. E. (2002). Pine oil cleaner-resistant *Staphylococcus aureus*: reduced susceptibility to vancomycin and oxacillin and involvement of SigB. *Appl Environ Microbiol* **68**, 5417–5421.

Russell, A. D. (1999). Bacterial resistance to disinfectants: present knowledge and future problems. *J Hosp Infect* **43** (Supplement), S57–S68.

Russell, A. D. (2002). Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria. *Symp Ser Soc Appl Microbiol* 31, 121S–135S.

Russell, A. D., Tattawasart, U., Maillard, J. Y. & Furr, J. R. (1998). Possible link between bacterial resistance and use of antibiotics and biocides. *Antimicrob Agents Chemother* **42**, 2151.

Rutala, W. A., Stiegel, M. M., Sarubbi, F. A. & Weber, D. J. (1997). Susceptibility of antibiotic-susceptible and antibiotic-resistant hospital bacteria to disinfectants. *Infect Control Hosp Epidemiol* **18**, 417–421.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U. & Vahaboglu, H. (2003). Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol* **52**, 403–408.

Schwartz, L. S., Jansen, N. B., Ho, N. W. Y. & Tsao, G. T. (1988). Plasmid instability kinetics of the yeast S288C pUCKm8 [Cir(+)] in non-selective and selective media. *Biotechnol Bioeng* **32**, 733–740.

Stecchini, M. L., Manzano, M. & Sarais, I. (1992). Antibiotic and disinfectant susceptibility in Enterobacteriaceae isolated from minced meat. *Int J Food Microbiol* **16**, 79–85.

Suller, M. T. E. & Russell, A. D. (2000). Triclosan and antibiotic resistance in *Staphylococcus aureus*. *J Antimicrob Chemother* **46**, 11–18.

Tabata, A., Nagamune, H., Maeda, T., Murakami, K., Miyake, Y. & Kourai, H. (2003). Correlation between resistance of *Pseudomonas aeruginosa* to quaternary ammonium compounds and expression of outer membrane protein OprR. *Antimicrob Agents Chemother* **47**, 2093–2099.

Thorrold, C. A., Letsoalo, M. E., Duse, A. G. & Marais, E. (2007). Efflux pump activity in fluoroquinolone and tetracycline resistant *Salmonella* and *Escherichia coli* implicated in reduced susceptibility to household antimicrobial cleaning agents. *Int J Food Microbiol* **113**, 315–320.

To, M. S., Favrin, S., Romanova, N. & Griffiths, M. W. (2002). Postadaptational resistance to benzalkonium chloride and subsequent physicochemical modifications of *Listeria monocytogenes*. *Appl Environ Microbiol* **68**, 5258–5264.

Edited by: A. R. Walmsley