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Resistance to phenicol compounds following adaptation to quaternary ammonium compounds in *Escherichia coli*

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ABSTRACT

Bacterial adaptation to quaternary ammonium compounds (QACs) is mainly documented for benzalkonium chloride (BC) and few data are available for other QACs. The aim of this study was to assess the effects of repeated exposure to different quaternary ammonium compounds (QACs) on the susceptibility and/or resistance of bacteria to other QACs and antibiotics. *Escherichia coli* strains ($n = 10$) were adapted by daily exposure to increasingly sub-inhibitory concentrations of a QAC for 7 days. Three QACs were studied. Following adaptation, we found similar levels of reduction in susceptibility to QACs with a mean 3-fold increase in the minimum inhibitory concentration (MIC) compared to initial MIC values, whatever the QAC used during adaptation. No significant differences in antibiotic susceptibility were observed between the tested QACs. Antibiotic susceptibility was reduced from 3.5- to 7.5-fold for phenicol compounds, β lactams, and quinolones. Increased MIC was associated with a shift in phenotype from susceptible to resistant for phenicol compounds (florfenicol and chloramphenicol) in 90% of *E. coli* strains. Regardless of the QAC used for adaptation, exposure to gradually increasing concentrations of this type of disinfectant results in reduced susceptibility to QACs and antibiotics as well as cross-resistance to phenicol compounds in *E. coli* strains. Extensive use of QACs at sub-inhibitory concentrations may lead to the emergence of antibiotic-resistant bacteria and may represent a public health risk.

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1. Introduction

Disinfectants are major compounds used in the food production chain during the steps of cleaning and disinfection to ensure microbiologically safe food products. Classified as biocides, they are commercialized as active substances alone or in combination. In the process of reviewing (Biocide Directive 98/8/EC), they must be evaluated for their potential unacceptable effects on target organisms, such as unacceptable resistance and cross-resistance. There are currently few data on resistance and cross-resistance following the use of biocides. Through standard *in vitro* bactericidal suspension tests, disinfectants have generally been demonstrated to be efficient in eliminating bacteria in food (Van de Weyer et al., 1993). However, in field conditions, bacteria are regularly exposed to sub-lethal concentrations of disinfectants and this may constitute a selective pressure driving the acquisition of resistant genes or adaptation of initially susceptible bacteria (Hegstad et al., 2010). This situation may be responsible for decreased susceptibility or resistance to not only commonly used disinfectants, but also to other biocides or even antibiotics used in human therapy. Resistance to biocides has only been documented for a limited number of biocides, such as triclosan and benzalkonium chloride and, in most cases, has been studied on few bacterial strains, making it impossible to

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conclude whether biocide resistance is strain-dependent or not (Langsrud et al., 2004). Results of studies on the potential link between the use of biocides and decreased susceptibility or resistance to antibiotics are controversial. Scientific evidence is therefore insufficient for correctly assessing the risks of biocide and antibiotic resistance as reported by the Scientific Committee for Emergent and Newly Identified Health Risks (SCENIHR, 2009). Regarding QACs, studies in over the past several years have been mainly done on adaptation to benzalkonium chloride (BC) in different bacteria species (Aase et al., 2000; Braoudaki and Hilton, 2004). Hence, to compensate for the lack of data on decreased susceptibility and potential resistance to antibiotics following bacterial adaptation to biocides, our aims were to assess the impact of two QACs (didecyl dimethyl ammonium chloride (DDAC) and dioctyl dimethyl ammonium chloride (OCDAC)) commonly used in the food industry and compare them to BC in 10 *Escherichia coli* strains.

2. Materials and methods

2.1. Strains

Nine different previously characterized avian and porcine *E. coli* strains and a reference strain *E. coli* ATCC®25922 were used in this study. They were selected from strains collected as part of the annual antibiotic resistance monitoring programs organized by the French Ministry of Agriculture. Table 1 lists the origin of these strains and their susceptibility to antimicrobial compounds commonly used in human and veterinary medicine and the minimum inhibitory concentration (MIC) value for BC. *E. coli* ATCC®25922 (AM10) was also used as quality-control strain for each antibiotic-susceptibility test (CLSI, Clinical and Laboratory Standards Institute 2008, M31-A3). The strains were kept at −80 °C in a storage nutritive solution (0.5% tryptone, 0.3% bovine extracts and 15% glycerol). Strains were spread with a loop on Mueller-Hinton (MH) agar (Becton Dickinson, Le-Pont-de-Claix, France) and incubated 24 h at 37 °C.

2.2. Disinfectants and antibiotics

The disinfectants used in this study are quaternary ammonium compounds (QACs) and are the most commonly used biocide formulations in the French agri-food industry. They included benzalkonium chloride (BC, BTC50, Stepan Europe, Voreppe, France), didecyl dimethyl ammonium chloride (DDAC, Bardac22, Lonza Bâle, Switzerland), dioctyl dimethyl ammonium chloride (OCDAC, Bardac LF, Lonza Bâle, Switzerland). Disinfectant stock solutions contained 50% of the tested QAC as the biocidal active substance. Customized microtiter plates containing dilution ranges of dehydrated antibiotics were purchased from Trek Diagnostic Systems (East Grinstead, England). The following antibiotics were included: ampicillin (AMP), ceftazidime (TAZ), cefotaxime (FOT), chloramphenicol (CHL), ciprofloxacin (CIP), florfenicol (FFN), gentamicin (GEN), nalidixic acid (NAL), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMP).

2.3. Antibiotic and disinfectant susceptibility tests

Antibiotic susceptibility tests were performed using the microdilution method with the Sensititre® system on the customized microtiter plate. Two or three *E. coli* colonies from MH agar were suspended in sterile demineralized water and this suspension was adjusted to a 0.5 McFarland standard (bioMérieux, Marcy-l'Etoile, France). This bacterial suspension (10 μl) was diluted in 10 ml of MH broth and 50 μl were automatically inoculated by the Sensititre® in each well of the microtiter plates. *E. coli* ATCC®25922 (AM10) was used as a quality-control strain for each antibiotic-susceptibility test. After incubation for 24 h at 37 °C, bacteria growth was assessed by observing turbidity in the medium. The strains were interpreted as susceptible or resistant to antibiotics according to the epidemiological resistance cut-off determined from EUCAST and CA-SFM guidelines for *Enterobacteriaceae*. These cut-offs (breakpoint concentrations in μg/ml) are >2 (FOT); >8 (TAZ); >16 (NAL); >8 (TET); >16 (CHL); >4 (GEN); >16 (STR); >8 (TMP); >256 (SMX); >8 (AMP); >16 (FFN); >1 (CIP). Culture purity was checked by streaking 1 μl of suspension used for plate inoculation on MH agar supplemented with 5% (v/v) defibrinated sheep blood (AES Laboratoires, Combourg, France). Viable bacteria were enumerated from 100 μl of inoculation suspension diluted to 1/200 on MH agar and the value had to be in the range from 25 to 80 CFU/ml to validate the test.

Table 1
Antibiotics and BC susceptibilities for the studied *E. coli* strains.

Strain	Origin	Year sampled	MIC BC (μg/ml)	Antibiotic susceptibility profile
AM01	Pork	2005	32	Susceptible
AM02	Pork	2004	32	TET
AM03	Pork	2005	32	TET
AM04	Pork	2000	16	TET-STR-SMX
AM05	Poultry	2002	32	Susceptible
AM06	Poultry	2002	32	Susceptible
AM07	Poultry	2002	16	TET-STR-SMX
AM08	Poultry	2001	16	NAL
AM09	Poultry	2004	16	AMP-SMX
AM10	Control strain		32	Susceptible

TET: resistant to tetracycline; STR: resistant to streptomycin; SMX: resistant to sulfamethoxazole; NAL: resistant to nalidixic acid; AMP: resistant to ampicillin.

For validation, MIC values for antibiotics in the quality-control *E. coli* strain had to fall within the MIC ranges reported in the CLSI-M31-A3 guidelines (CLSI 2008).

The MIC for disinfectant, corresponding to the lowest concentration of disinfectant that prevents bacterial growth, was determined using a standard macrodilution method. From stock solutions of 50% (equivalent to 50×10^4 μg QAC/ml) of each QAC disinfectant (BC, DDAC, OCDAC), dilutions of disinfectant were prepared in distilled water to obtain the concentrations as follows: 10–15–20–30–40–60–80–120–160–240–320–480–640–960–1280–1920–2560 μg QAC/ml. From these concentrations, a specific concentrations range was used according the QAC tested for BC from 40 to 640 μg/ml, for DDAC from 10 to 160 μg/ml and for OCDAC from 320 to 2560 μg/ml. One ml of each QAC concentration of this range was added to 10 ml of MH broth containing overnight bacterial suspension (10^5 CFU/ml). After incubation for 24 h at 37 °C, bacteria growth was assessed by observing turbidity in the medium

All determinations of MIC for QAC and antibiotics were repeated on two different days. If the results from the first two tests differed beyond one dilution, a third assay was done.

2.4. Adaptation experiments: exposure to quaternary ammonium compounds

Adaptive responses of *E. coli* strains were investigated by exposing the strains daily to increasing concentrations of each QAC (BC, DDAC, OCDAC) for 7 days. Two or three *E. coli* colonies were suspended in MH broth and incubated for 24 h at 37 °C. The suspension was then diluted 100-fold and incubated for 2 h before calibrating it to an OD_{600} value (spectrophotometer Model 254, Sherwood, Cambridge, UK) of 0.1 resulting in 10^8 CFU/ml. This calibrated bacterial suspension (100 μl) was initially exposed to a starting concentration of disinfectant below the MIC (0.5 × MIC) for 24 h at 37 °C in a total volume of 10 ml of MH broth. The disinfectant concentration range was prepared as described above for disinfectant susceptibility testing. When growth was observed, a 10-fold diluted culture was transferred to fresh MH broth supplemented with a higher concentration of disinfectant. If no growth was observed, the previous concentration was used. As control, a bacterial suspension (100 μl) and MH broth (10 ml) without disinfectant were tested using the same protocol. After 7 days, bacteria were spread with a loop (10 μl) on MH agar and incubated for 24 h at 37 °C. They were collected with 2.5 ml of storage nutritive solution and kept in cryotubes at −80 °C. For each QAC compound, adaptation experiments were repeated on two different days. MIC increase factor to each QAC was determined for each strain as a ratio between MIC after adaptation and MIC before adaptation.

2.5. DNA fingerprinting

Strains before and after exposure to QAC were characterized using Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) to confirm strain continuity.

PCR amplifications were carried out in a final volume of 50 μl containing 0.5 μmol/l of each primer: ERIC1-R and ERIC2 (Versalovic et al., 1991), 0.2 mmol/l dNTP, 2 mmol/l $MgCl_2$, Go *Taq* Flexi buffer 1X (Promega, Charbonnières-les-Bains, France), 1.25 U of Go*Taq* Flexi and 5 μl of DNA extracted using Instagene™ Kit (Biorad, Marnes-la-Coquette, France) from bacteria cultured overnight in MH broth. The PCR conditions involved an initial denaturation at 95 °C for 10 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 4 min and a final extension at 72 °C for 8 min. Amplifications were performed in a 9700 GenAmp thermocycler (Applied Biosystems, Les Ulis, France).

PCR products were separated by electrophoresis for 3 h at 110 V on 1% standard agarose gel (Sigma, St. Quentin Fallavier, France) containing 1X TAE (40 mmol/l Tris, 40 mmol/l acetate, 1 mmol/l EDTA pH 8.3) buffer, stained with ethidium bromide (0.5 μg/ml) for 15 min and viewed under UV light. Images were recorded with Biocapt software (Vilber Lourmat, Torcy, France).

2.6. Statistical analysis

The effects of bacterial adaptation to disinfectant on increases in MIC for disinfectants and antibiotics were analyzed using ANOVA. Variances homogeneity was verified using the Levene test. The analyses were carried out using Systat 13 (Systat Software, WA, USA) and *p*-values <0.05 were considered to be significant.

3. Results

3.1. Experiments on adaptation to quaternary ammonium compounds

Before the adaptation experiments, MIC values for a given QAC varied among *E. coli* strains from 16 to 32 μg/ml for BC, 1.5 to 3 μg/ml for DDAC and 32 to 128 μg/ml for OCDAC (data not shown). For each *E. coli* strain, the modification of susceptibility for QAC following adaptation was determined by calculating MIC increase factor. Adaptation process resulted in an increase in MIC whatever the studied QACs (Fig. 1). The mean increase of MIC was slightly but not significantly ($p < 0.05$) higher in strains adapted to DDAC (mean increase in MIC, 3.5-fold) compared to the same strains adapted to the two other QACs (mean increase in MIC, 2.6–2.9-fold).

3.2. Effect of adaptation to QACs on reduced susceptibilities to other QACs

After exposure to increasing sub-inhibitory concentrations of one QAC, strains were screened for susceptibility to the two other QACs (Table 2). In all cases, strains exhibited similar reduced susceptibility to both QACs ($p < 0.05$). Indeed, the highest mean increases in MIC were found for DDAC after exposure of the strains to the two other QAC molecules: 3.4-fold increase with OCDAC and 2.9-fold increase with BC.

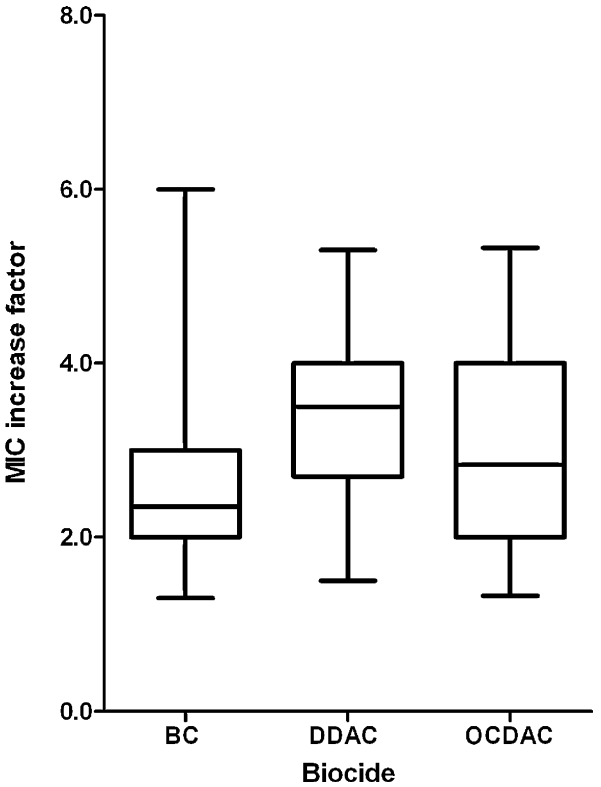

Fig. 1. Plots showing MIC increase factor to benzalkonium chloride (BC), didecyl dimethyl ammonium chloride (DDAC), and dioctyl dimethyl ammonium chloride (OCDAC) after adaptation to each of these molecules in *E. coli* strains. Values are box plots (first and third quartiles with minimum and maximum values, $n = 10$). Solid lines represent mean values of MIC increase factor.

3.3. Effects of QAC adaptation on reduced susceptibility and resistance to antibiotics

The effects of QAC adaptation were measured by comparing mean MIC increase for antibiotics between strains before and after adaptation to QACs. From the statistical analysis using the Student–Newman–Keuls test ($p < 0.05$), five groups (denoted as A through E) of antibiotics were identified (Fig. 2). The highest effect was found for phenicol compounds (FFN, CHL) with about 8-fold mean increase in MIC values following adaptation with DDAC and OCDAC. A less pronounced effect was noted for a cephalosporin molecule (FOT) with a mean increase of 6-fold. For many molecules from different antibiotic families (TAZ, TET, NAL, CIP, TMP, AMP), susceptibilities decreased about 4-fold. No effect was found on aminosides (GEN, STR) and SMX. Significant variation in antibiotic MIC values between strains was observed, as revealed by standard deviations. Overall, the effects of QAC adaptation on reduced susceptibility to antibiotics were similar for the three QAC compounds used in adaptation experiments. No adaptation effect was noted for control strains exposed in MH broth without QACs as indicated by MIC increases of about one-fold (Fig. 2).

After exposure to QACs, MIC increases sometimes led to a modification in susceptibility with strains initially susceptible to antibiotics becoming resistant. In these cases, the MIC value was superior to the epidemiological threshold MIC value given by antimicrobial testing committees. This was in particular the case for CHL and FFN (Table 3) with nine out of ten strains resistant to FFN following adaptation to at least one of the studied QACs. All strains resistant to CHL were resistant to FFN. In a few cases, modification of the antibiotic susceptibility profile was observed for tetracycline (strain AM10) and ampicillin (strain AM07).

Table 2
Mean MIC increases (*n*-fold) for QACs after exposure to increasing concentrations of each QAC in 10 *E. coli* strains.

Exposure to:	Mean increases in MIC (±SD) for:		
	BC	DDAC	OCDAC
BC		2.9 (±0.9)	1.8 (±0.7)
DDAC	2.6 (±0.9)		2.1 (±0.6)
OCDAC	2.7 (±0.9)	3.4 (±1.4)	

BC: benzalkonium chloride; DDAC: didecyl dimethyl ammonium chloride; OCDAC: dioctyl dimethyl ammonium chloride; SD: standard deviation.

3.4. DNA fingerprinting

For each strain, ERIC-PCR profile was identical before and after adaptation whatever the studied QAC, even for parent strains and strains exposed in MH broth without disinfectant as controls (data not shown).

4. Discussion

Disinfectant use is currently being called into question because the regular use of these substances in food production may select for bacteria that are less susceptible to biocides and antibiotics due to bacterial adaptation. This adaptive response may be of major concern in practical conditions where bacteria are in contact with low or residual disinfectant concentrations. To our knowledge, the present study is the first to compare the effects of repeated exposure to three different QACs: BC, dimethyl didecyl ammonium chloride (DDAC), and dimethyl dioctyl ammonium chloride (OCDAC) on the susceptibility and/or resistance to QACs and antibiotics in 10 *E. coli* strains. The results showed that the QAC used for adaptation did not influence the magnitude of adaptive response to that QAC because similar mean (roughly 3-fold) increases in MIC values to other QACs were obtained after the adaptation experiment. In two *E. coli* reference strains, Langsrud et al. (2004) also found reduced susceptibility to BC by about 3-fold after the same adaptation time of 7 days. The highest increase (6-fold) in MIC for BC was detected after a longer period of adaptation (24 days), reaching a final MIC for BC of 150 μg/ml. Although our adaptation protocol was somewhat different (daily inoculation in this study *vs.* up to 3 days of culture before re-inoculation), we found similar results after 7 days of adaptation (Fig. 1). This indicates that our protocol based on a shorter exposure time of bacteria to a given disinfectant concentration is not

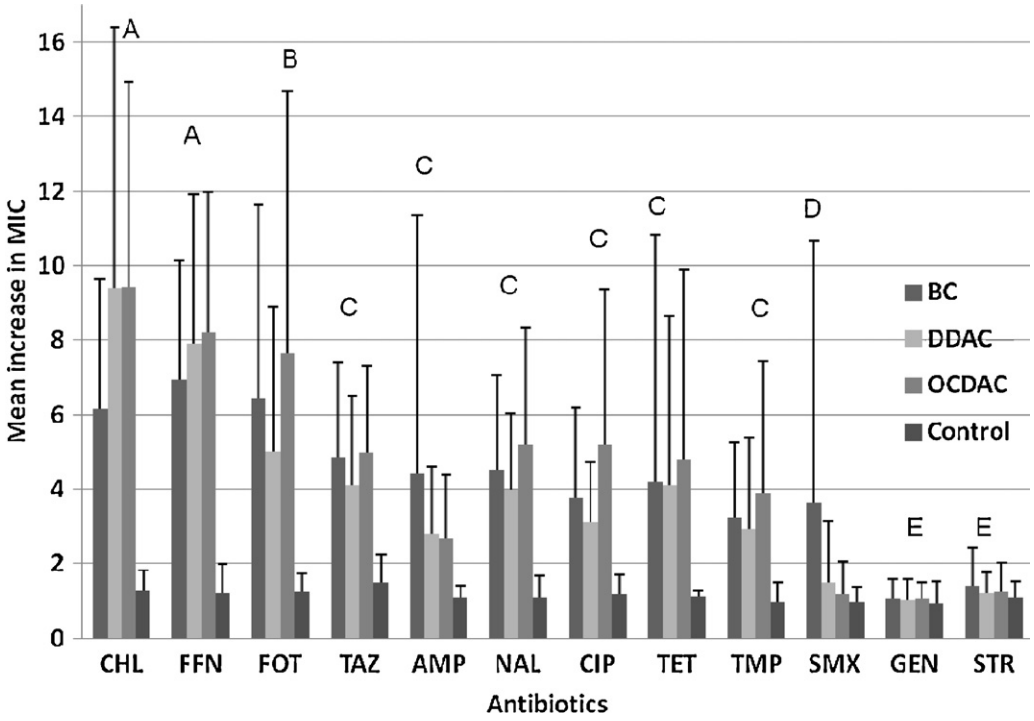

Fig. 2. Mean increase in MIC to antibiotics observed after exposure to benzalkonium chloride (BC), didecyl dimethyl ammonium chloride (DDAC), dioctyl dimethyl ammonium chloride (OCDAC) and to culture broth without disinfectant (control) in 10 *E. coli* strains. MIC tests were done twice. Letters (A–E) over each group of tested QACs (excluding the control) indicate a statistically significant difference among antibiotics ($p < 0.05$). Error bars represent standard deviation.

detrimental because a similar level of reduced susceptibility to QAC was obtained. All 10 *E. coli* strains showed adaptation to QACs, suggesting that adaptation is not strain-dependent. Identical ERIC-PCR profiles were found before and after adaptation confirming strain continuity across the adaptation experiments.

All QACs were shown to cause cross-adaptation among QACs. Furthermore, adaptation to one QAC did not lead to higher cross-adaptation to other QACs, as indicated by similar increases in MIC (Fig. 1). This result is in agreement with similar adaptation levels in *Listeria monocytogenes* strains adapted to QAC and amine compounds (Lunden et al., 2003) leading to the speculation that the adaptation mechanism is probably non-specific. It is possible that this was the case for the QACs used in this study.

Table 3
Escherichia coli strains resistant to antibiotics (R) following adaptation to BC, DDAC and OCDAC.

Strain	Adaptation to:	Antibiotics			
		CHL	FFN	TET	AMP
AM01	BC–DDAC–OCDAC		R		
AM02	BC–DDAC	R	R		
	OCDAC		R		
AM03	BC–DDAC–OCDAC	R	R		
AM04	OCDAC	R	R		
AM05	BC–DDAC	R	R		
AM06	BC–OCDAC	R	R		
	DDAC		R		
AM07	OCDAC	R	R		R
AM08	DDAC–OCDAC	R	R		
AM10	BC	R	R		
	DDAC–OCDAC	R	R	R	

R: strains found to be resistant in both experiments.

The impact of BC adaptation on reduced susceptibility and resistance depended on the antibiotic families tested on the *E. coli* strains. The greatest reduction in susceptibility (8-fold) associated with a change in the antibiotic susceptibility profile (susceptible to resistant) according to epidemiological resistance thresholds was for phenicol compounds (CHL, FFN) in practically all *E. coli* strains (9 out of 10). This result was supported by the Langsrud et al. (2004) study in which the level of response towards CHL was higher (12–24-fold) for the two *E. coli* reference strains studied, although no information on their susceptibility or resistance to CHL was provided. In another study (Braoudaki and Hilton, 2004), *E. coli* O157 H7 adapted to BC exhibit cross-resistance not only to phenicol compounds, but also to other antibiotics (TMP, TET, AMP). Differences in antibiotic resistance observed between our non-pathogenic *E. coli* strains for which the serogroup was unknown and *E coli* O157 H7 could be explained by variation in antibiotic resistance according to serotype or serogroup as previously demonstrated in *Salmonella* and *E. coli* (Braoudaki and Hilton, 2004).

Two strains were found to be resistant to antibiotics after adaptation to at least one QAC: one to TET and the other to AMP (Table 2). However, their MIC values were

just one dilution above the epidemiological threshold. Adaptation to BC also influenced susceptibility up to 4-fold to other antibiotics such as quinolones (CIP, NAL), β lactams (AMP, TAZ, FOT) and tetracyclins (TET), but had no impact on susceptibility to aminosides (GEN, STR). In contrast to our results, increased tolerance to these antibiotics has been shown for *E. coli* K-12 gradually adapted to BC (Bore et al., 2007). It is noteworthy that even if the magnitude of the increase in MIC to antibiotics was lower (4-fold), this increase may be a first step in acquired resistance to antibiotics and may contribute to higher resistance in combination with other factors, such as biofilms. Further study on the effects on antimicrobial susceptibility in other bacteria species could be done from one out of these three QAC (BC, DDAC, OCDAC) as we found susceptibility profiles to antibiotics and biocides identical whatever the QAC used for adaptation. However, other studies have demonstrated contrasting results and adaptation to QAC does not always result in reduction of antibiotic susceptibility or resistance. This is particularly true for bacteria isolates derived from *in situ* experiments. For instance, hospital isolates of *Pseudomonas aeruginosa* adapted to BC are not less susceptible to antibiotics and other biocides (Loughlin et al., 2002). In contrast, a relationship has recently been found between high QAC MICs and resistance to one or more antibiotics in Gram-negative bacteria isolated from community settings (Carson et al., 2008). Therefore, it would be interesting to further investigate the impact of biocide use on the reduced susceptibility or resistance of bacteria to antibiotics in conditions close to those used in everyday practice using biofilms microcosm as models. The effects observed at the level of individual strains exposed to QAC *in vitro* might be different compared to those observed on bacteria community present in biofilms (Moore et al., 2008).

The mechanisms involved in the adaptive response to QAC in this study are not identified but seem to be non-specific as reduced susceptibility is observed for antibiotics having different mechanisms of action and for QAC. The adaptive response to QAC was accompanied by the expression of efflux pumps. The efflux pumps activity were inhibited in presence of efflux inhibitor (phe-arg-β-naphtylamide) and resulted in increased sensitivity to BC in theses field strains (data not shown). AcrAB efflux pump positively regulated by transcriptional activators (MarA, SoxS) was demonstrated to export biocides (triclosan, chlorhexidine, QAC) and multiple antibiotics (Levy, 2002). This efflux pump could also contribute to QAC resistance but other pumps or membrane proteins (OmpC, OmpF) as well (Poole, 2005). Further experiments by constructing *marA* and *acrAB* mutants could be done to verify the contribution of the genes.

Bacteria with increased MIC to disinfectants are rarely resistant to concentrations actually used (Aase et al., 2000; Langsrud and Sundheim, 1997). However, decreased susceptibility to disinfectants may represent a selective advantage for bacterial survival in biofilms, where the adapted bacteria are in competition with other bacteria species. Adapted bacteria may protect the most sensitive bacteria as recently demonstrated by Lee et al. (2010), where, by producing a signalling molecule, a few highly resistant *E. coli* mutants improved the survival of more vulnerable bacteria in stressful conditions. This hypothesis would be interesting to test in experiments using biofilms as a model, simulating field conditions as much as possible.

In conclusion, misuse of QAC-based disinfectants resulting in exposure of bacteria to sub-inhibitory concentrations could lead to the emergence of antibiotic-resistant bacteria isolates and therefore represent a public health risk.

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