Bacterial Biofilms Resist Oxidising Agents Due to the Presence of Organic Matter

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Abstract

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This study evaluated the susceptibility of planktonic and biofilm cells of *Staphylococcus* spp. (*n* = 87), *Klebsiella* spp. (*n* = 30), and *Escherichia coli* (*n* = 74) isolates originating from food contact surfaces of milk and meat processing plants to benzalkonium chloride (BAC), sodium hypochlorite (NaClO), chloramine B (CAB), and peracetic acid (PAA). Bacterial growth and reduction of viable cells in the presence of disinfectants were determined in tryptone soya broth (TSB) and water, respectively. Biofilm positive isolates (*n* = 73) were tested for the presence of selected *qac* genes. Unlike BAC, chlorine-based disinfectants and PAA were poorly efficient in TSB, especially in the case of biofilms. However, when tested in water, the efficacy of NaClO, CAB and PAA substantially increased, which was particularly evident in biofilms. In water, staphylococcal biofilms were even more susceptible to CAB than planktonic cells. A part (23.3%) of the biofilm positive staphylococci carried the *qac* genes but did not express an increased resistance to BAC. This study showed that bacterial biofilms protected with organic matter could be one of the main reasons for disinfection failure.

Keywords: food safety; MIC; dairy; sanitation; biocide; hygiene

Microorganisms occurring in the food industry could be a source of secondary contamination for edible products (Flint *et al.* 1997; Sharma & Anand 2002). Moreover, their attachment to food contact surfaces and survival in the form of microbial communities, known as biofilms, pose a risk for the occurrence of long-persisting contaminated sites on food processing equipment (Zottola & Sasahara 1994). Some bacteria (i.e. the genera *Klebsiella, Pseudomonas* and *Staphylococcus*) produce exopolymers that can facilitate adhesion of other microorganisms which can survive in the form of mixed biofilms (Sasahara & Zottola 1993). Both pathogenic and food spoilage microorganisms have been isolated from such bacterial communities, which may occur on sites difficult to identify (e.g. interior surfaces, crevices, dead spaces) and where organic material providing protection and nutrients to microorganisms can also accumulate (Simoes *et al.* 2010). Therefore, an efficient sanitation program is a critical precondition for the production of safe food.

Use of disinfectants is an inseparable part of the sanitation procedure in the food industry. Over

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time, however, an increasing resistance to disinfectant agents has been observed among various bacterial species (Langsrud *et al.* 2003). Several studies have found that *Listeria monocytogenes* and *Enterobacter aerogenes* or bacteria of the genera *Bacillus*, *Streptococcus*, *Staphylococcus*, *Shigella*, *Escherichia*, and *Klebsiella* are able to survive cleaning and disinfection (Austin & Bergeron 1995; Sharma & Anand 2002; Gunduz & Tuncel 2006). Moreover, several genes, such as *qac* and *smr* in staphylococci or *sug*E in *Escherichia coli*, have so far been described to confer the resistance to quaternary ammonium compounds (Heir *et al.* 1999; Chung & Saier 2002). It is also well recognised that bacteria persisting in a biofilm community are much more resistant to biocidal agents than planktonic cells of the same species (Carpentier & Cerf 1993; Campanac *et al.* 2002) and that the presence of organic material greatly impairs the efficacy of some disinfectants (Aarnisalo *et al.* 2000). Due to this fact, conventional sanitation and disinfectant agents may fail to kill bacteria under certain conditions (Hodd & Zottola 1997).

The aim of this study was to test selected disinfectant agents commonly used in the food industry against a range of *Staphylococcus* spp., *Klebsiella* spp. and *E. coli* isolates. Disinfectant agents were tested in a growth medium (thus in the presence of organic matter supporting the growth) as well as in water. In these two media, the tendency of efficacy of different types of disinfectants (oxidising vs. surface active) was studied on biofilm and planktonic cells of different types of bacteria (Gram-positive vs. Gram-negative). A special emphasise was laid on the capability of biofilms to survive and release bacterial cells into a growth supporting medium under the pressure of particular disinfectants.

MATERIAL AND METHODS

Bacterial isolates. A total of 191 isolates (Table 1), obtained from food contact surfaces in milk and meat processing plants within two hours after sanitation, were collected from 2005 to 2009. Isolates of the same species and from the same plant differed in the site and/or date of isolation. The isolates (stock cultures) were kept at –80°C in nutrient broth No. 2 (Oxoid, Basingstoke, UK) supplemented with 20% glycerol. Bacterial suspensions prepared from the second subculture (37°C; 20 h) onto tryptone soya agar (Oxoid, Basingstoke, UK) were used in the tests. For the quality control purposes, the reference strains *Staphylococcus aureus* ATCC 6538 and *E. coli* ATCC 10536 were included.

Disinfectant agents and neutralisers. Benzalkonium chloride (BAC, 95%; Sigma Aldrich, Brondby, Denmark), sodium hypochlorite (NaClO, 11%; Penta, Strakonice, Czech Republic), chloramine B (CAB 28%; Penta, Strakonice, Czech Republic) and peracetic acid (PAA 38–40%; Merck, Darmstadt, Germany) were tested in twofold dilution ranges of 1.22–1250, 31.7–6500, 13.7–7000, and 4.88–40 000 μg/ml, respectively. The disinfectant agents were appropriately diluted in tryptone soya broth (TSB; Oxoid, Basingstoke, UK) or water, as indicated below, and immediately used in the tests. Neutralisers were prepared in 0.25 mol/l phosphate buffer. For neutralization of BAC, a 3% polysorbate 80 and 0.3% lecithin (Sigma-Aldrich, Steinheim, Germany) solution was used. The remaining disinfectants were neutralised with 0.5% sodium thiosulfate (Penta, Strakonice, Czech Republic). The neutralisers were validated as recommended by the European Standard EN 1276:1997. All the tests were performed in 96-well polystyrene microtitration plates (BD Falcon U-Bot-

Table 1. Bacterial isolates and their origin

	Dairy farms (n = 4)	Dairy plants (n = 3)	Meat plants (n = 2)	Poultry plants (n = 1)	Total
S. aureus	9 (6)[a]	5 (2)	6 (2)	2 (2)	22 (12)
S. epidermidis	39 (20)	19 (9)	5 (1)	2 (0)	65 (30)
K. pneumoniae	6 (5)	8 (8)	1 (0)	1 (0)	16 (13)
K. oxytoca	3 (1)	7 (1)	2 (2)	2 (0)	14 (4)
E. coli	12 (1)	7 (0)	34 (8)	21 (5)	74 (14)
Total	69 (33)	46 (20)	48 (13)	28 (7)	191 (73)

[a]the numbers of biofilm positive isolates are indicated in parenthesis

tom Tissue Culture Plates; BD-Biosciences, Erembodegem, Belgium).

Biofilm production. An overnight TSB culture of test microorganisms was diluted (1:100) with fresh TSB for *E. coli* and *Klebsiella* spp. biofilms or with TSB supplemented with 1% glucose and 1% NaCl (TSBgn) for *Staphylococcus* spp. biofilms. Two hundred microliters of the diluted cultures were dispensed into each well of the microtitration plate and incubated at 37°C for 48 hours. After 24 h of incubation, the medium was replaced with the appropriate fresh medium (TSB or TSBgn). The biofilms were semi-quantified by crystal-violet staining (Stepanović *et al.* 2000). Only the isolates that produced strong biofilms (with $OD_{570} \geq 1.4$, which was at least three times higher than OD of biofilm-negative isolates) were selected for the testing of biofilm susceptibility.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). Minimum inhibitory concentrations for planktonic cells (P-MIC) were determined in TSB by the broth microdilution method according to the approved standard of the Clinical and Laboratory Standards Institute (CLSI document M07-A8). One well per each isolate contained TSB without disinfectants and served as a growth control. One column of each microtitration plate contained only TSB and served as a control of sterility. Each isolate was tested in duplicate. After incubation at 37°C for 20 h, P-MICs were determined spectrophotometrically (OD_{605}, Sunrise Absorbance Reader; Tecan, Salzburg, Austria) and 10 µl from each well was then transferred into the corresponding wells of a microtitration plate prefilled with 90 µl of a suitable neutralising solution. After neutralisation for 5 min at 20°C, 10 µl from each well was transferred into the corresponding wells of a new microtitration plate prefilled with 90 µl of fresh TSB and incubated (37°C, 20 h) to determine minimum bactericidal concentrations (P-MBC).

To determine minimum inhibitory concentrations for biofilms (B-MIC), these were incubated (37°C; 20 h) in 250 µl of TSB containing appropriately diluted disinfectants. Each isolate was tested in duplicate including growth and sterility control wells. After incubation, 100 µl from each well was transferred into a new microtitration plate and B-MICs were determined spectrophotometrically (see above). B-MICs were therefore considered as those concentrations of disinfectants that inhibited release and/or growth of bacterial cells released from a biofilm structure. The disinfectants in biofilms were then neutralised with 250 µl of a suitable neutralising solution for 5 min at 20°C. After neutralisation, biofilms were sonicated in 250 µl of fresh TSB at 110 W for 30 min (ultrasonic bath 9LE; Kraintek, Hradec Kralové, Czech Republic) and incubated at 37°C (20 h) to determine minimum bactericidal concentrations (B-MBC).

Minimum efficient concentration (MEC). The lowest concentrations of disinfectants which reduced the number of viable cells by ≥ 5 log were considered as MECs. MECs for planktonic (P-MEC) and biofilm (B-MEC) cells were determined in water for 50 selected isolates. To determine P-MEC, the suspension test EN 1276:1997 was modified for application to microtitration plates. Briefly, a bacterial suspension in distilled water containing 0.1% tryptone (Oxoid, Basingstoke, UK) and 0.85% NaCl was incubated (20°C, 2 min) with a 0.3% water solution of bovine albumin (Merck, Darmstadt, Germany) at a ratio of 1:1 (simulation of clean conditions) and then 20 µl of the suspension was dispensed into microtitration plate wells prefilled with 80 µl of hard water containing appropriately diluted disinfectants. The final number of bacteria ranged between 2 and 4×10^6 CFU per well. Each isolate was tested in duplicate. The bacteria were incubated with disinfectants for 5 min at 20°C. After neutralisation as described above, 10 µl of neutralised bacterial suspension was transferred into 90 µl of fresh TSB and incubated at 37°C for 20 hours. The lowest concentration of disinfectants which led to the negative bacterial cultivation in TSB was determined as P-MEC (reduction of ≥ 5 log).

To determine B-MEC, 200 µl of hard water containing appropriately diluted disinfectants was added to the biofilms pre-incubated (20°C, 2 min) with 50 µl of a 0.15% water solution of bovine albumin. Each isolate was tested in duplicate including two wells per isolate with biofilms exposed only to water (non-treated control). After incubation for 5 min at 20°C, the wells were neutralised as described above. After neutralisation, the biofilms were sonicated in 250 µl of TSB as described above and 100 µl of the sonicated bacterial suspension was then transferred into the corresponding wells of a microtitration plate prefilled with 150 µl of fresh TSB. The microtitration plate was then incubated at 37°C in the Sunrise Absorbance Reader. To draw the bacterial growth curves, absorbances (OD_{605}) were measured every 15 min during 20 h of incu-

bation. The numbers of viable cells from biofilms were estimated through the calibration growth line experimentally prepared for each isolate as described below. A reduction in the number of viable cells is expressed as a delta value between treated and non-treated biofilms.

Calibration growth lines. To draw the calibration growth line, serial tenfold bacterial dilutions (10^1–10^8 CFU/ml) were incubated in 250 µl of TSB at 37°C in the Sunrise Absorbance Reader. The absorbance (OD_{605}) was measured every 15 min during 20 hours. For each the isolate we obtained eight growth curves as shown in Figure 1. Empirical growth curves were fitted by a model (sigmoid) curve (Zwietering *et al.* 1990):

$$\text{absorbance}\ (t) = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + \exp[-(t - t_i)/b]}$$

where:

t – time in h

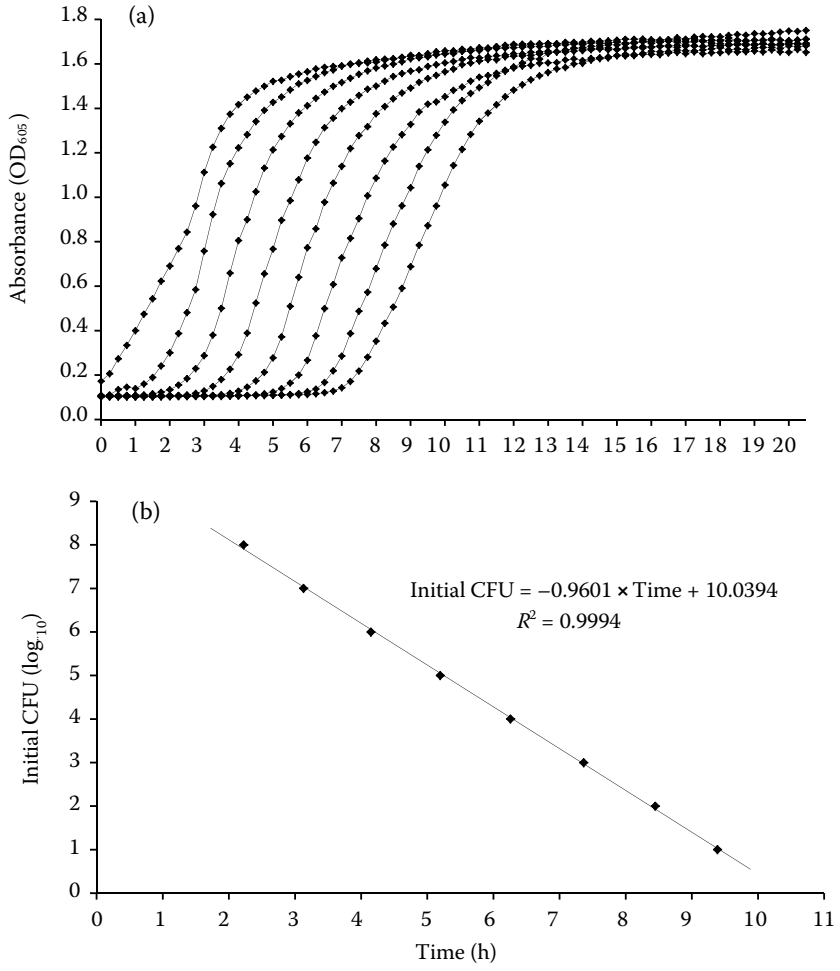
absorbance (t) – absorbance in time t

bottom, top, t_i, b – model parameters

For each growth curve we determined the start time and the end of the exponential phase of growth (by the method of the second derivative maximum), the inflection point of the growth curve and the time at which the absorbance level reached 0.8. The time corresponding to the absorbance of 0.8 was in a close linear dependence with the initial number of bacterial cells as demonstrated by the coefficients of determination ($R^2 > 0.9$). Therefore, using the method of linear regression, the time required for reaching the absorbance of 0.8 could be assigned to the initial number of bacterial cells through the calibration growth line (Figure 1).

The calibration growth line was calculated for each isolate from three independent experiments. Based on the analysis of variance (ANOVA), the variability among individual experiments was not significant ($P > 0.05$) and the mean decrease of one log with 95% confidence interval was determined for each isolate (the range of confidence interval was < 0.1 h).

PCR. Biofilm positive isolates (n = 73; Table 1) were tested by PCR for the presence of genes en-



(a) Growth curves corresponding to the serial tenfold dilutions of 10^1–10^8 CFU/ml (from right to left); (b) Calibration growth line showing a linear dependence between the time at which the absorbance of 0.8 is reached and the initial number of bacterial cells; R^2 – coefficient of determination

Figure 1. An example of the calibration growth line drawn for *Staphylococcus aureus* isolate 1491

coding for the resistance to quaternary ammonium compounds (QACs) and some other cationic biocides. PCR was carried out for the detection of *qac*A/B, *qac*G, *qac*H, *qacJ* and *smr* (formerly *qac*C) genes as described previously (Bjorland *et al.* 2001, 2005; Anthonisen *et al.* 2002). *Staphylococcus haemolyticus* NVH97A (Bjorland *et al.* 2005) and *S. aureus* RN4220 (Bjorland *et al.* 2003) were used as positive control strains.

RESULTS AND DISCUSSION

It is known that organic matter, which may accumulate at highly risky sites such as crevices, pipe joints, blind ends etc. (Simoes *et al.* 2010), can greatly affect the efficacy of disinfectant agents (Wirtanen & Salo 2003; Lourenco *et al.* 2009). In such an organic load microorganisms can long persist, grow and consequently be released into food products (Schlegelová *et al.* 2010). A case of multiple human intoxication caused by a toxigenic strain of *S. aureus* persisting on the interior surface of dairy plant equipment was reported in Japan (Asao *et al.* 2003). Therefore, by testing a large number of various isolates in the same study, we tried to bring more light into this problem taking into account several factors such as medium tested (growth medium vs. water), disinfectant types (surface active vs. oxidising) and the cell form (planktonic vs. biofilm cells). In order to evaluate the potential of selected disinfectant agents to inhibit or kill bacteria in the presence of organic matter we determined both MICs and MBCs in TSB although the purpose of disinfection is to kill (not inhibit) bacteria and disinfection at 37°C for 24 h is not relevant for practical conditions. Because neither MIC nor MBC values established in growth medium reflect those concentrations of disinfectants that should appropriately reduce the number of microorganisms during short-run disinfection (Cerf *et al.* 2010), we also determined MECs which should lead to the reduction of viable cells by ≥5 log under conditions relevant for practice provided that the disinfected equipment is clean.

The MIC and MBC values for both planktonic and biofilm cells are shown in Tables 2 and 3. Among the isolates, the highest variability in susceptibility was observed with BAC. Especially in Gram-positive bacteria (i.e. staphylococci), B-MICs of BAC were higher than P-MICs and even P-MBCs. B-MICs could be interpreted as concentrations which impede a release of bacterial cells from the biofilm structure, and also as those inhibiting the growth of the released cells. Therefore, the fact that B-MICs of BAC were higher than P-MICs may indicate an increased resistance of bacterial cells released from biofilms. This may be a consequence of physiological alterations of

Table 2. Minimum inhibitory and bactericidal concentrations (μg/ml) determined in TSB for planktonic cells. Concentrations of NaClO and CAB are expressed as concentrations of free chlorine as evaluated according to the standard EN ISO 7393-3:1990

	Staphylococcus aureus		*Staphylococcus epidermidis*		*Escherichia coli*		*Klebsiella* spp.	
	P-MICs	P-MBCs	P-MICs	P-MBCs	P-MICs	P-MBCs	P-MICs	P-MBCs
BAC	2.44–9.77[a] (4.88/4.88)[c]	4.88–19.5[b] (19.5/19.5)[d]	1.22–9.77 (4.88/2.44)	4.88–19.5 (19.5/9.77)	19.5–39.1 (39.1/39.1)	19.5–156.3 (78.1/78.1)	19.5–78.1 (39.1/19.5)	19.5–156.3 (78.1/39.1)
NaClO	2031–4063 (4063/2031)	2031–4063 (4063/4063)	2031–4063 (4063/2031)	2031–4063 (4063/4063)	2031–4063 (4063/2031)	2031–4063 (4063/2031)	1016–2031 (2031/2031)	1016–2031 (2031/2031)
CAB	437.5–875 (875/875)	875	437.5–875 (875/875)	437.5–875 (875/875)	875	875–1750 (1750/875)	437.5–875 (875/875)	437.5–875 (875/875)
PAA	625	625–1250 (1250/625)	312.5–625 (625/625)	625–1250 (625/625)	625–1250 (625/625)	625–1250 (1250/625)	625	625–1250 (1250/625)

P-MICs – minimum inhibitory concentrations determined for planktonic cells; P-MBCs – minimum bactericidal concentrations determined for planktonic cells; BAC – benzalkonium chloride; NaClO – sodium hypochlorite; CAB – chloramine B; PAA – peracetic acid

[a]P-MIC range; [b]P-MBC range; [c]P-MIC inhibiting 90% of isolates/P-MIC most frequently occurred; [d]P-MBC killing 90% of isolates/P-MBC most frequently occurred

Table 3. Minimum inhibitory and bactericidal concentrations (μg/ml) determined in TSB for biofilm cells. Concentrations of NaClO and CAB are expressed as concentrations of free chlorine as evaluated according to the standard EN ISO 7393-3:1990

	Staphylococcus aureus		*Staphylococcus epidermidis*		*Escherichia coli*		*Klebsiella* spp.	
	B-MICs	B-MBCs	B-MICs	B-MBCs	B-MICs	B-MBCs	B-MICs	B-MBCs
BAC	39.1–78.1[a] (78.1/78.1)[c]	156.3–625[b] (625/312.5)[d]	19.5–156.3 (78.1/39.1)	78.1–625 (312.5/156.3)	39.1–156.3 (78.1/78.1)	78.1–625 (625/312.5)	39.1–156.3 (78.1/78.1)	156.3–625 (625/312.5)
NaClO	2031–4063 (4063/2031)	> 65 000	2031–4063 (4063/2031)	> 65 000	2031–4063 (4063/4063)	> 65 000	2031–4063 (4063/4063)	> 65 000
CAB	875	> 7 000	875–1750 (875/875)	> 7 000	875–1750 (875/875)	> 7 000	875–1750 (875/875)	> 7 000
PAA	625–1250 (1250/1250)	> 40 000	625–1250 (1250/625)	> 40 000	625	> 40 000	1250	> 40 000

B-MICs – minimum inhibitory concentrations determined for biofilm cells; B-MBCs – minimum bactericidal concentrations determined for biofilm cells; BAC – benzalkonium chloride; NaClO – sodium hypochlorite; CAB – chloramine B; PAA – peracetic acid

[a]B-MIC range; [b]B-MBC range; [c]B-MIC inhibiting 90% of isolates/B-MIC most frequently occurred; [d]B-MBC killing 90% of isolates/B-MBC most frequently occurred

biofilm cells (Boulange-Petermann 1996) or the result of the pressure of sublethal disinfectant concentrations (Russell 2003).

In the remaining disinfectants, all the biofilms survived extremely high concentrations (Table 3) and P-MICs, P-MBCs and B-MICs were comparable among each other and among the isolates of the same and different species (Tables 2 and 3). This could be explained by the high instability of these agents in TSB. Due to rapid inactivation, sublethal concentrations stop being effective and initially inhibited but survived bacterial cells can start to grow (P-MICs were similar to P-MBCs). Nevertheless, in spite of the fact that all biofilms survived the highest tested concentrations, the B-MICs of these disinfectants were also at the level of P-MBCs. Therefore, it is apparent that some other nonspecific factors (such as adverse pH, changed nutrient properties of the medium due to the oxidative reactions, a certain degree of cell damage and survival of cells only in deep biofilm layers) may affect viability, release from biofilms and growth of bacterial cells.

As demonstrated in planktonic cells, CAB was shown to be more efficient in TSB than NaClO although combined available chlorines (i.e. chloramines) were described to be less effective than hypochlorites, which belong to a group of free available chlorines (Rutala & Weber 1997). This could be explained by the higher stability of chloramines (Kim *et al.* 2002) suggesting that these disinfectants are more appropriate than free available chlorines for the disinfection of contaminated environments. In contrast to chlorine-based disinfectants and PAA, the influence of TSB on the efficacy of BAC did not seem to be so significant since MIC and MBC values were relatively low, especially in the case of planktonic cells. Moreover, similar concentrations of BAC were found to inhibit planktonic staphylococci in both TSB (this study) and Mueller-Hinton broth (Sundheim *et al.* 1992). These showed higher BAC stability indicating that exposure time, which is one of the fundamental factors influencing the efficacy of disinfection (Cerf *et al.* 2010), may play a more important role in this disinfectant than the level of organic contamination.

Although we realize that the tests in TSB and water were performed under different conditions and, therefore, the results cannot be compared, there is no doubt that the different types of disinfectants (i.e. stable BAC vs. unstable chlorines and PAA) had a completely different tendency of efficacy in these two different media. In contrast to BAC where the MEC values were generally higher than those determined in TSB as MICs or MBCs, in all other disinfectants the MEC values were much lower despite the much shorter exposure time (Tables 4 and 5). In water, resistance to disinfectants generally increased in biofilms

Table 4. Minimum efficient concentrations determined in water for planktonic cells of 50 selected isolates (P-MECs in μg/ml). Concentrations of NaClO and CAB are expressed as concentrations of free chlorine as evaluated according to the standard EN ISO 7393-3:1990

	Staphylococcus aureus (*n* = 12)		*Staphylococcus epidermidis* (*n* = 13)		*Escherichia coli* (*n* = 13)		*Klebsiella* spp. (*n* = 12)	
BAC	39.1–78.1[a]	78.1/78.1[b]	39.1–78.1	78.1/39.1	78.1–312.5	312.5/156.3	78.1–312.5	312.5/156.3
NaClO	127–253.9	127/127	127–253.9	253.9/127	127–253.9	253.9/253.9	127	127
CAB	54.7–109.4	109.4/109.4	54.7–109.4	109.4/109.4	54.7–109.4	109.4/54.7	54.7–109.4	109.4/54.7
PAA	19.5–39.1	39.1/39.1	19.5	19.5	9.8–39.1	19.5/19.5	19.5–39.1	39.1/39.1

BAC – benzalkonium chloride; NaClO – sodium hypochlorite; CAB – chloramine B; PAA – peracetic acid
[a]P-MEC range; [b]P-MEC for 90% isolates/P-MEC most frequently occurred

Table 5. Minimum efficient concentrations determined in water for biofilm cells of 50 selected isolates (B-MECs in μg/ml). Concentrations of NaClO and CAB are expressed as concentrations of free chlorine as evaluated according to the standard EN ISO 7393-3:1990

	Staphylococcus aureus (*n* = 12)		*Staphylococcus epidermidis* (*n* = 13)		*Escherichia coli* (*n* = 13)		*Klebsiella* spp. (*n* = 12)	
BAC	312.5–1250[a]	1250/625[b]	156.3–1250	625/312.5	312.5–1250	625/312.5	312.5–1250	625/312.5
NaClO	507.8–2031	2031/2031	253.9–2031	2031/507.8	507.8–2031	2031/2031	253.9–2031	2031/1016
CAB	27.3–54.7	54.7/27.3	27.3–54.7	54.7/27.3	27.3–109.4	109.4/54.7	27.3–109.4	109.4/109.4
PAA	9.8–78.1	78.1/78.1	9.8–78.1	78.1/19.5	9.8–78.1	78.1/39.1	625–2500	1250/1250

BAC – benzalkonium chloride; NaClO – sodium hypochlorite; CAB – chloramine B; PAA – peracetic acid
[a]B-MEC range; [b]B-MEC for 90% isolates/B-MEC most frequently occurred

compared to planktonic cells but this phenomenon was not as obvious as in TSB. When expressed in relation to the planktonic cells, different levels of biofilm resistance can be observed between different disinfectants. B-MECs were markedly higher than P-MECs in BAC (except for the Gram-negative bacteria) and NaClO. On the other hand, with the exception of *Klebsiella* spp., resistance of biofilms to PAA was not so high when compared with planktonic cells. Finally, CAB was shown to be most efficient against biofilms with surprisingly lower B-MECs than P-MECs in *Staphylococcus* spp. (Tables 4 and 5).

It was observed in both TSB and water that planktonic cells of Gram-positive bacteria were more susceptible to BAC than those of Gram-negative bacteria (Tables 2 and 4). However, the susceptibility of planktonic and biofilm cells was similar between Gram-positive and Gram-negative bacteria regardless of the medium tested. It was already stated that Gram-positive bacteria are generally more susceptible to biocides than Gram-negative bacteria. This could be due to the cell wall structure in Gram-positive bacteria which may facilitate the penetration of biocides, and due to the outer membrane of Gram-negative bacteria which may impede the entry of biocides into the cell (Russell 2003). In this study, however, an increased resistance of *E. coli* and *Klebsiella* spp., compared to that of staphylococci, was observed only with BAC and not with other disinfectants. Similarly, Wirtanen & Salo (2003) in their review underlined ineffectiveness against Gram-negative bacteria in QACs but not in peracetic acid and chlorines. These indicate that not only the permeability of the cell wall or outer membrane but also other factors (i.e. a mechanism of biocide action) may be responsible for such differences in bacterial susceptibility. In other words, benzalkonium chloride, a member of QACs, affects

the cytoplasmic membrane, which is a mechanism of action quite different from that described in halogens (i.e. chlorine) and peroxygens (i.e. peracetic acid) (McDonnell & Russell 1999). A higher susceptibility of staphylococci to BAC (compared to Gram-negative bacteria) observed only in planktonic but not in biofilm cells may indicate that the ability of biofilm formation may compensate the intrinsic susceptibility of Gram-positive bacteria to QACs.

The testing of disinfectants in water showed that biofilms should not always be implicitly considered as a highly resistant bacterial form. A relatively high resistance of bacterial biofilms, when compared with planktonic cells, was clearly observed only with BAC and NaClO. The fact that B-MECs of BAC were only slightly higher than P-MECs in Gram-negative bacteria could be logically explained by their intrinsic resistance to BAC. However, an unexpected but not unique finding was the relatively low resistance of bacterial biofilms to PAA and especially to CAB. A similar phenomenon has also been reported by some other authors when planktonic rather than biofilm cells demonstrated a higher resistance to BAC and PAA for *S. aureus* and *Pseudomonas aeruginosa*, respectively (Spoering & Lewis 2001; Cabo *et al.* 2009). Superior penetration of chlorosulfamates (a group of disinfectants to which CAB also belongs) into biofilms was already reported (Stewart *et al.* 2001) although, unlike the present study, the increased susceptibility of biofilms to these disinfectants was not observed. It seems that the stationary phase of bacterial growth as well as nutrient limitations are the main reasons why planktonic cells may be similarly or even more resistant to some biocides than biofilms (Spoering & Lewis 2001; Anderl *et al.* 2003). The whole issue, however, appears to be more complex since, in contrast to staphylococci and *E. coli*, biofilms produced by *Klebsiella* spp. were highly resistant to PAA in the present study. Therefore, the level of biofilm resistance may also depend on bacterial species involved in biofilm production.

Seven biofilm positive *S. epidermidis* isolates (23.3%) harboured one of the *qac* genes (5 isolates had the *qac*A/B gene and 2 isolates had the *smr* gene). However, increased resistance to BAC was not observed in these isolates when compared with PCR negative isolates (data not shown). Conversely, Smith *et al.* (2008) reported significantly higher resistance to QACs in *S. aureus* isolates carrying the *qac* genes when compared to isolates without these genes. Between these two groups of the isolates the authors, however, did not observe any significant differences in susceptibility to another cationic biocide – chlorhexidine. Therefore, the factors influencing the expression of the various Qac pumps remain to be further explained.

According to Russell (2003), concentrations of disinfectants for sanitation are generally higher than those experimentally determined as inhibitory or lethal. For example, concentrations of BAC and chlorines usually recommended for disinfection of environmental surfaces are 200 and 100–5000 ppm, respectively (Sundheim *et al.* 1992; Rutala & Weber 2004). In general, such concentrations seem to be sufficient for the elimination of most bacteria occurring in planktonic forms and on clean surfaces but this cannot be assumed for biofilms or highly contaminated sites.

CONCLUSION

The results of this study showed that in the presence of organic matter bacterial biofilms can tolerate extremely high concentrations of oxidising agents such as chlorines and PAA. On the other hand, a relatively high efficacy of PAA and especially CAB against bacterial biofilms may be expected on clean surfaces.

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