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Original article

Resistance of *Escherichia coli* growing as biofilms to disinfectants

C Ntsama-Essomba[1], S Bouttier[1], M Ramaldes[1], F Dubois-Brissonnet[2], J Fourniat[1]*

[1] *Laboratoire de microbiologie industrielle, faculté de pharmacie, 5 rue JB Clément, 92296 Châtenay-Malabry cedex;*
[2] *Laboratoire de microbiologie industrielle, Ensia, 91305 Massy cedex, France*

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Summary — The bactericidal activity of various disinfectants (cationic or amphoteric surfactants, oxidizing agents, phenolic derivatives) was determined against *Escherichia coli* CIP 54127 obtained by culture on tryptic soy agar (in-suspension or on-germ-carrier test) or in the form of biofilms produced in a continuous culture system. The bacteria tested on germ-carriers or included in biofilms were more resistant than the same strain in suspension. The extent of the reduction in activity depended on the nature of the disinfectant. In the two cases, the greatest reduction was observed with benzalkonium chloride and hexadecyl trimethylammonium bromide, the agents with the lowest hydrophile-lipophile balance. The activity of the oxidizing agents (sodium hypochlorite, peracetic acid/H_2O_2) and alkyl trimethylammonium derivatives (C12 and C14) was somewhat reduced, while that of the phenolic derivatives (*o*-cresol, phenol) was either slightly attenuated or unaffected. The reduction in sensitivity was attributed to a reduced accessibility of the bacterial cells to the disinfectants, due to the fact that the former adhered to a support. Furthermore, the interfering action of the substances in contact with the bacteria (milk in the germ-carrier test and exopolymers in the biofilms) could play a role. The reduced sensitivity of the bacteria in the biofilms was not due to any alteration in the metabolic state of the bacteria (mostly in a quiescent state) since this resistance was lost after the mechanical resuspension of the cells before the contact with the disinfectants.

***Escherichia coli* / biofilm / disinfectant / germ-carrier**

Résumé — Résistance aux désinfectants de *Escherichia coli* obtenu sous forme de biofilms. L'activité bactéricide de divers désinfectants (agents de surface cationiques ou amphotères, agents oxydants et dérivés phénoliques) a été déterminée vis-à-vis de cellules de *Escherichia coli* CIP 54127 obtenues par culture sur gélose trypticase soja (essais en suspension et sur porte-germes) ou sous forme de biofilms obtenus dans un système en culture continue. Les bactéries étudiées selon la méthode porte-

* Correspondence and reprints
Tel: (33) 01 46 83 55 45; fax (33) 01 46 83 55 45.

germes ou incluses dans le biofilm étaient plus résistantes que les bactéries étudiées en suspension. L'importance de la réduction d'activité dépend de la nature du désinfectant. Dans les deux cas, les ammoniums quaternaires de faible balance hydrophile-lipophile (benzalkonium et hexadécyl triméthyl ammonium) ont été les substances dont l'activité bactéricide a été la plus affectée. L'activité des agents oxydants (hypochlorite de sodium, acide peracétique/H_2O_2) et des alkyl (C12 et C14) triméthyl ammoniums était réduite à un moindre degré. Celle des dérivés phénoliques (*o*-crésol, phénol) était peu ou pas affectée. Cette diminution de sensibilité semble être surtout la conséquence d'une moindre accessibilité des cellules aux désinfectants liée à l'adhésion des bactéries aux supports, à laquelle pourrait s'ajouter la présence de substances interférentes localisées au contact des bactéries (lait dans l'essai porte-germes, exopolymères dans le cas des biofilms). Lorsque les bactéries du biofilm étaient mécaniquement remises en suspension avant l'essai du désinfectant, leur résistance disparaissait, suggérant que la moindre sensibilité des bactéries incluses dans le biofilm n'est pas liée à une résistance propre des bactéries, conséquence de leur état métabolique (cellules pour la plupart dans un état quiescent).

***Escherichia coli* / biofilm / désinfectant / porte-germes**

INTRODUCTION

In an aquatic medium, a few microorganisms live in the planktonic state, and most adhere to submerged surfaces forming complex communities bound by excreted exopolymers (Costerton et al, 1987). The initial adhesion to a hard-surface, a reversible process, is the resultant of physico-chemical interactions (surface charges, hydrophobicity) between the outer surfaces of the microorganisms and the supports (Bryers, 1987). Production of exopolymers may render this adhesion irreversible and the immobilized cells then multiply and continue to secrete exopolymers leading to a colonization of the surface in the form of a biofilm (Costerton et al, 1987). In a natural medium, biofilms consist of the bacteria and the matrix of exopolymers along with other substances or particles (mineral or organic, other microorganisms) that have come in contact with the matrix (Characklis and Marshall, 1990). Growth in the form of a biofilm has ecological advantages as it renders the cells more resistant to environmental influences (flow, toxic substances, radiation, desiccation, bacteriophages), and microbial biofilms represent a common source of contamination in industrial and medical fields. Furthermore, biofilms are frequently more difficult to kill and eliminate than their homologs in suspension (Costerton et al, 1987; Maris, 1992; Ntsama-Essomba et al, 1995; Dubois-Brissonnet et al, 1995). There are a variety of situations where the resistance of microorganisms in biofilms has economic, environmental and public health consequences, as biofilms may be encountered in water cooling circuits, metal working processes, drinking water distribution circuits, foodstuff manufacturing plants and medical implants (Costerton et al, 1987). The mechanism behind the resistance of biofilms is not yet well understood. Various hypotheses have been advanced including a protective action of the matrix, alterations in the physiological state of the bacteria, or neutralization of anti-microbial substances within the matrix. In the present study, mono-microbial biofilms of *Escherichia coli* were obtained in a continuous culture system. We compared the sensitivity of the biofilms to various disinfectants from different chemical classes to that of the same bacterial strain obtained by culture on tryptic soy agar.

MATERIALS AND METHODS

Bacterial strain

The bacterial strain selected for the tests was *Escherichia coli* CIP 54127 (ATCC 10536), a Gram-negative strain used in French Standards AFNOR (anonymous, 1995a) and selected by a working group of the European Committee for Normalisation (CEN TC 216) for the evaluation of the activity of antiseptics and disinfectants (anonymous, 1995b).

This strain was maintained according to the French Standard AFNOR T 72-140 (anonymous, 1995a). For inoculation into the flow system or to determine its susceptibility to disinfectants, it was used after three successive inoculations on tryptic soy agar (TS agar) slants (Merck) at 24 h intervals at 37 °C.

Formation of biofilms

As described by Le Magrex et al (1994), the biofilms were obtained in a loop of PVC tubing (6.5 mm internal diameter and 1.94 m long) (Tygon® Masterflex, Bioblock, Paris, France) in a continuous flow reactor. The medium (60 mL) in the loop was maintained in circulation (flow rate: 240-250 mL/min) with a peristaltic pump (Masterflex). Medium was added continuously with a second peristaltic pump at a flow rate of 2.5 mL/min.

The medium introduced into the loop was a minimum medium containing: 0.1 g casamino acid peptone (Difco), 0.1 g yeast extract (Difco), 1.25 g anhydrous Na_2HPO_4, 0.5 g KH_2PO_4, 0.25 g lactose, 0.2 g $MgSO_4.7\ H_2O$, and 0.5 mg $FeSO_4.7\ H_2O$, per litre distilled water. This medium was prepared and sterilized (20 min at 121 °C) without $MgSO_4$, which was added in sterile 100-fold concentrated solution before use.

With this system the maximal viable adherent population (1-2.10^8 CFU/cm^2) was obtained 48 h after inoculation. This population remained relatively stable over the following 8 days of observation. The thickness of the biofilm (estimated by scanning electron microscopy) increased linearly: 3-4 µm at 48 h, reaching 12-15 µm by day 10.

Evaluation of the cellular metabolic activity by flow cytometry

The metabolic activity of the 5-day-old biofilm bacteria was evaluated by flow cytometry and compared with that of 18-24-h-old bacteria obtained on TS agar slants or in suspension in the exponential growth phase (5-h culture in tryptic soya broth under magnetic stirring) used as control for metabolically active population. The biofilm bacteria were resuspended by scraping and vortexing in phosphate buffer saline (8.5 g NaCl, 3.3 g $Na_2HPO_4.12\ H_2O$, and 1.1 g KH_2PO_4, per litre distilled water, pH 7.2) (PBS). The bacteria obtained on agar were suspended in PBS. The bacteria obtained in broth were centrifuged (2 500 *g* for 10 min) and resuspended in PBS. The bacteria in suspension (adjusted to about 10^7 bacteria/mL) were incubated with fluorassure viability marker (Chemchrom®, Chemunex, Maisons-Alfort, France). The labelled cells were then injected into a flow cytometer (Chemflow® Autosystem II, Chemunex), which detected and counted individual fluorescent cells. The fluorescent signals emitted by the bacteria were automatically classified according to their intensity, which was proportional to the metabolic activity of the cells.

Bactericidal activity of disinfectants

The bactericidal activity of the following nine disinfectants was evaluated: benzyldimethyl tetradecylammonium chloride (benzalkonium chloride) (Fluka), hexadecyl trimethylammonium bromide (Sigma), tetradecyl trimethylammonium bromide (Sigma), dodecyl trimethylammonium bromide (Sigma), amphoteric surfactants derived from lauric acid in solution at 20% w/V active matter (Tegol® 2000, Goldschmidt, Montigny-le-Bretonneux, France), sodium hypochlorite solution at 39.0 ± 0.6 g/L available chlorine (Colgate-Palmolive R&D, Courbevoie, France), peracetic acid used as a 2.5% w/V solution with 18% w/V hydrogen peroxide (Bactipal®, Seppic, Paris, France), *o*-crésol (Sigma) and phenol (Prolabo, Paris, France). For the assays, the agents were diluted in sterile distilled water just before use. The test concentration range was (in % w/V or V/V according to the physical state (solid or liquid) of the disinfectant under test): 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, etc.

Tests were conducted on the bacteria obtained on TS agar slants (on-carrier or in-suspension tests), the bacteria in biofilms or the biofilm bacteria mechanically resuspended.

Determination of bactericidal activity against bacteria cultured on TS agar slants

The bactericidal activity of the nine disinfectants was determined according to the method of the French Standard Afnor T 72-300 (in-suspension test). For at least one representative of each category of disinfectant, assays were also conducted according to French Standard Afnor NF T 72-190 (on-germ-carrier method) (anonymous, 1995a).

Briefly, for the T 72-300 standard, dilutions of disinfectant in distilled water were incubated with the microorganisms in suspension ($1\text{-}3 \times 10^7$ CFU/mL) at 20 °C for 5 min ± 5 s. The action of the agent was then stopped by the transfer of 1 mL of the mixture to 9 mL of neutralizing diluent. After 10 min ± 5 s contact time at 20 °C, two samples of 1 mL were transferred to Petri dishes and included in plate count agar (PCA) (Merck). The neutralizing diluents selected after validation were as follows: for benzalkonium chloride, alkyl trimethylammonium derivatives, *o*-crésol and phenol: a solution of polysorbate 80 at 5% V/V, with 5% V/V fresh egg yolk added before use; for amphoteric biocides: neutralizing diluent described in the French Pharmacopoeia (anonymous, 1993); for sodium hypochlorite: 0.5% w/V sodium thiosulfate solution; and for peracetic acid/H_2O_2: a solution of peroxidase (30 IU/100 mL) (Merck) and ascorbic acid (0.5% w/V) (Merck) in pH 7.0 phosphate buffer (3.75 g KH_2PO_4, 6.97 g $Na_2HPO_4.2H_2O$ per litre distilled water). Polysorbate 80 solution, French Pharmacopoeia neutralizing diluent, thiosulfate solution and phosphate buffer were sterilized over 20 min at 120 °C in an autoclave.

For the NF T 72-190 standard, 0.05 mL of a bacterial suspension (at a concentration sufficient to obtain around 10^6 CFU after drying on the carrier) in diluent containing 5% V/V of reconstituted skimmed milk (Merck) were deposited on the carrier (polyethylene plate). After drying for 45 min at 37 °C, the inoculum was covered with 0.2 mL of the dilution of disinfectant (assay) or distilled water (control) for 5 min ± 5 s. Then the carrier was transferred into 100 mL neutralizer (1 g tryptone Difco, 8.5 g NaCl, 3 g polysorbate 80 per litre distilled water, sterilized for 20 min at 120 °C), and after 1 min sonication, the number of viable bacteria were counted as described in the relevant standard.

For each test, the minimal bactericidal concentration (MBC) corresponded to the lowest concentration which killed 99.999% (5 log reduction) of the viable bacterial cells under specified conditions. The validity of the results was checked according to the procedures laid down in the relevant standards.

Determination of bactericidal activity against the bacteria included in the biofilms and resuspended biofilm bacteria

For the bacteria included in biofilms, the bactericidal activity of the disinfectants was determined using the methodology of the T 72-300 standard, except that the inoculum in suspension was replaced by a 2 cm sample of tube with biofilm and 1 mL of distilled water. Briefly, a 2 cm section of loop with biofilm was immersed in distilled water to remove planktonic bacteria, then plunged into 10 mL of disinfectant diluted in distilled water. After 5 min ± 5 s, the tube was removed and immersed in 10 mL of neutralizer for 10 min at 20 °C. During these 10 min, the biofilm was detached by sonication for 1 min and vortexing for 30 s. Two samples of 1 mL and two samples of 0.1 mL of the suspension were placed in Petri dishes with PCA. To determine the biofilm population before the treatment with disinfectant, another portion of tube with biofilm was treated in the same manner, except that disinfectant was replaced by distilled water and the suspension obtained in neutralizer was diluted 10^{-5} before seeding. After 48 h incubation, the colonies were counted and the population before and after disinfectant treatment were calculated for 1 cm^2 of tube.

Other assays were conducted with different durations of contact in order to determine, for a given concentration, the minimal time which induced a 5 log reduction in the biofilm population.

To determine the activity of the disinfectants towards bacteria released from biofilms prior to disinfection, the biofilms were detached from the tube samples by scraping with a Pasteur pipette and vortexing in tryptone salt diluent (1 mL of diluent for 2 cm of tube with biofilm)

for 1 min. The bacterial suspension obtained was used as the inoculum in the assay according to the T 72-300 standard.

The same neutralisers were used after validity as described in the previous paragraph.

RESULTS

Metabolic activity of biofilm bacteria

Compared with the bacterial population harvested in the exponential growth phase, the population of the 5-day-old biofilms consisted mainly of slightly active cells with only a small fraction exhibiting marked metabolic activity (fig 1). The population of bacteria sampled from the agar culture was moderately active.

Bactericidal activity of disinfectants

Bactericidal activity of disinfectants against bacteria on agar

For the bacteria obtained from the TS agar slants, there was a marked decrease in activity against the bacteria on polyethylene plates (Afnor NF T 72-190) with respect to that determined in suspension (Afnor T 72-300) for all disinfectants tested apart from phenol (table I). The reduction was more marked for the long alkyl chain surfactants (> 12 C: MBC × 40-1000) than for the oxidizing agents (sodium hypochlorite, peracetic acid/H_2O_2) (MBC × 5-10).

Bactericidal activity of disinfectants against biofilm bacteria

Activity of disinfectants against 5-day-old biofilm cells

Only the activity of phenol was unaffected by the biofilm. Compared with their activity towards bacteria tested in suspension, the activity of the other disinfectants (oxidizing agents, cationic and amphoteric surfactants, *o*-cresol) was reduced to varying extents when bacteria were included in the biofilm (table I). The molecules with the highest molecular weights (benzalkonium chloride and hexadecyl trimethylammonium bromide) were most affected by the presence of the biofilm (more than 400-fold increase in MBC). For the three alkyl trimethylammonium derivatives, the reduc-

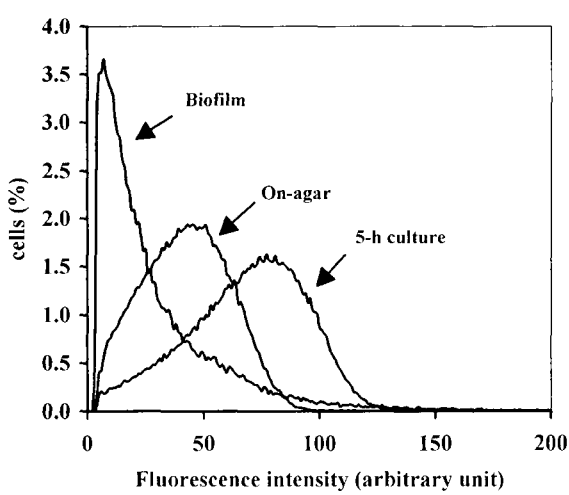

Fig 1. Metabolic activity (measured by flow cytometry) of *Escherichia coli* CIP 54127 cells obtained as a function of culture conditions : cells of 5-day-old biofilms, cells obtained on TS agar (18-24 h culture), cells in exponential phase obtained in TS broth (5 h culture) (mean of three independant assays).

Table I. Minimal bactericidal concentration of oxydative and phenolic agents, and of cationic and amphoteric surface agents towards *Escherichia coli* CIP 54127.

Disinfectants [a]	*Bacteria obtained on TSA*		*Bacteria of 5-day-old biofilm*		*HLB* [e]
	in-suspension test	*on-carrier test (PET)*	*in-biofilm test*	*in-suspension test*	
Sodium hypochlorite [b] (% V/V)	0.1	0.5	0.5	0.1	–
Bactipal ® [c] (% V/V)	0.02	0.2	0.5	0.02	–
Phenol (% w/V)	2	1	2	1	–
o-Cresol (% w/V)	0.5	ND	2	0.5	–
Benzalkonium chloride (% w/V)	0.002	1	2	0.005	5.48
ATAB C16 (% w/V)	0.005	> 2	> 2	0.005	8.80
ATAB C14 (% w/V)	0.005	0.2	0.2	0.005	9.75
ATAB C12 (% w/V)	0.02	0.1	0.2	0.02	10.70
Tégol® [d] (% V/V)	0.05	1	2	0.05	–

[a] duration of contact with bacteria of 5 min at 20 °C; [b] Javel water at 39.0 ± 0.6 g/L available chlorine; [c] Solution at 2.5% w/V of peracetic acid and 18% w/V of hydrogen peroxyde; [d] Solution at 20% w/V of lauryl propylen diamino acetic acid + lauryl propylen diamine; [e] HLB Hydrophile-Lipophile Balance, calculated according to the formula of Davies and Rideal (Myers, 1990); ATAB Alkyl trimethyl ammonium bromide.

tion in activity was a function of the length of alkyl chain and of their hydrophile-lipophile balance. For the oxidizing agents, the activity of the peracetic acid/H_2O_2 mixture was most reduced (MBC × 25) by the biofilm, while that of sodium hypochlorite was only reduced by a factor of 5.

The resistance of the bacteria in the biofilms was lost if the biofilm was dispersed in distilled water before testing. For all disinfectants tested, the resuspended bacteria had sensitivities not significantly different from that of the bacteria obtained on agar medium and tested in suspension.

Influence of contact duration on MBC towards biofilm

The MBCs of sodium hypochlorite and benzalkonium chloride were determined for various durations of contact. For sodium hypochlorite, a reduction of 5 log units in the viable population in the biofilm was observed after 20 min of contact with the concentration (0.1% V/V) producing the same reduction in 5 min for bacteria in suspension. On the other hand, for benzalkonium chloride, this reduction was not observed even after contact of 6 h with the effective concentration (0.002% w/V) for 5 min contact with bacteria in suspension.

Table II. Minimal bactericidal concentration of biocide agents towards *Escherichia coli* CIP 54127 obtained as biofilms.

Assays	*Benzalkonium chloride (% w/V)*	*Sodium hypochlorite* [a] *(% V/V)*	*Phenol (% w/V)*
Bacteria of 2-day-old biofilm			
- included in biofilm	1	0.5	2
- in suspension	0.002	0.1	1
Bacteria of 5-day-old biofilm			
- included in biofilm	2	0.5	2
- in suspension	0.005	0.1	1
Bacteria of 10-day-old biofilm			
- included in biofilm	10	5	2
- in suspension	0.005	0.1	2

[a] Javel water at 39.0 ± 0.6 g/L available chlorine.

Influence of age of biofilm on the activity of disinfectants

The activity of three disinfectants was investigated (table II). The 2- and 5-day-old biofilms had comparable sensitivity towards benzalkonium chloride, sodium hypochlorite and phenol. In contrast, the 10-day-old biofilms exhibited increased resistance to benzalkonium chloride and sodium hypochlorite (MBC > 5-10-fold than for 5-day-old biofilms) and there was no change in the sensitivity towards phenol. On dispersal of the biofilms in water, the bacteria regained their former sensitivity to the three disinfectants for all ages of biofilm.

DISCUSSION

The activity of disinfectants in vitro are currently tested either on microorganisms in suspension or on germ carriers. The most commonly used methods mix the microorganisms in suspension with a solution of the compound under test, in the presence or absence of interfering substances selected as a function of the field of application of the agent and the mode of use (Kelsey and Maurer, 1974; anonymous, 1987, 1995a, b; Van Klingeren, 1995; Bloomfield, 1995). These substances have varying influences on the activity of the disinfectant. For example, proteins have little influence on phenolic derivatives, but strongly interfere with sodium hypochlorite and quaternary ammonium salts with or without aldehydes (Chantefort and Druilles, 1984). Testing microorganisms in suspension does not simulate the action of the agent under true conditions of use very well, especially for microorganisms adhering to surfaces (Van Klingeren, 1995). Methods using germ carriers have thus been designed to provide a more accurate simulation of normal conditions of use. In these methods, microorganisms obtained by culture on agar or liquid media are placed and dried on a supporting surface (Best et al, 1990; Holah et al, 1990; anonymous, 1995a; Van Klingeren, 1995; Bloomfield, 1995). However marked discrepancies between the germicidal activity found in the laboratory and that observed in the field are not uncommon (Anwar et al, 1992; Johnston and Jones, 1995; Holah et al, 1990; Maris, 1992). This is accounted

for by the fact that microorganisms on supports (pipes, reservoirs, wet surfaces in industrial environments, implants, probes in hospital environments) are protected both by adhesion to the surface and by the presence of exopolymers excreted after adhesion during multiplication on the support and producing a coherent structure referred to as a biofilm (Ruseska et al, 1982; Costerton et al, 1983, 1987; LeChevalier et al, 1988a; Holah et al, 1990; Anwar et al, 1992; Brown and Gilbert, 1993; Vess et al, 1993). In the germ carrier tests, microorganisms can adhere to the support during the drying operation, but do not have enough time to secrete exopolymers.

In the present study, we examined the activity of nine disinfectants (five surfactants, two oxidizing agents and two phenolic derivatives) against *Escherichia coli* CIP 54127 according to the French Standard tests T 72-300 (in-suspension test) and NF T 72-190 (on germ-carrier test using polyethylene support) and against this bacteria included in 5-day-old biofilm. For a disinfectant of each type, bactericidal activity was determined as a function of biofilm age (2-, 5- or 10-day-old). A similar reduction in activity was observed with the germ carrier and the 2- and 5-day-old biofilm with respect to that observed in the test in suspension. The activity of the various disinfectants towards bacteria was not affected to the same extent by the biofilm. The surfactants, except for the C12 alkyl trimethylammonium derivative, were most inhibited. For the phenolic derivatives, there was a slight reduction in activity with *o*-cresol, although phenol itself retained its full activity relative to that observed in the test in suspension.

The reduced activity towards microorganisms on germ carriers is generally accounted for because of interference with substances from the suspension medium adsorbed onto the cells during the drying stage of the preparation (milk in NF T 72-190), or by restricted access of the disinfectant molecules to the bacteria bound to the support (Gélinas and Goulet, 1983; Druilles et al, 1986; Holah et al, 1990; Samrakandi et al, 1994). The resistance of the biofilms is attributed to protection provided by the exopolymeric matrix produced during colonization of the surface (poor penetration of the disinfectant into the matrix, neutralization of disinfectants within the matrix) and/or to the altered physiological state of the biofilm bacteria (slow growth) (Costerton et al, 1987; Nichols, 1989; Van der Wende et al, 1989; Brown and Gilbert, 1993; De Beer et al, 1994). Lock et al (1984) suggested that the polysaccharide matrix acts as a molecular sieve. This was consistent with our observation that the disinfectants with high molecular weight (cationic surfactants, amphoteric derivative of lauric acid) were more inhibited than the low molecular weight agents (sodium hypochlorite, peracetic acid/H_2O_2, *o*-cresol). However, this does not account for the lack of inhibition of the phenolic derivatives and the marked inhibition of the oxidizing agents. Chemical interactions between the disinfectant and matrix components may play a role, and would help account for the difference in behavior of the highly reactive oxidizing agents and phenol (low reactivity at pH 6.5, pH of the 2% m/V solution with 90% in the non-ionized form). The reaction of hypochlorite with the biofilm components (polymer matrix, cells, adsorbed substances), which may account for the persistence of biofilms in pipes carrying drinking water, has been the subject of numerous studies (LeChevalier et al, 1988a, b; Characklis, 1990; De Beer et al, 1994; Srinivasan et al, 1995). Monochloramine, which is less reactive than hypochlorite and less effective against planktonic bacteria, has been found to be more effective than hypochlorite towards bacteria in biofilms (LeChevalier et al, 1988a). The matrix of the biofilm formed from polysaccharides excreted by the bacteria is hydrophilic and

polyanionic (Allison, 1992); therefore, ionic and hydrophilic-hydrophobic interactions could account as well, at least in part, for the protective action of the biofilm. We found that the hydrophilic and anionic compounds (hypochlorite, peracetic acid/H_2O_2) were less inhibited than the long chain cationic compounds (benzalkonium chloride, alkyl trimethylammonium derivatives). For the cationic surfactants, the extent of the inhibition was found to be inversely related to the hydrophile-lipophile balance. Interactions between the disinfectant and the biofilm matrix tended to slow the diffusion of molecules into the biofilm. For sodium hypochlorite, this was indicated by a decrease in the minimal bactericidal concentration towards the intact biofilm with increasing duration of contact. The effective concentration for 5 min contact with bacteria in suspension only induced the same decrease in the initial population (5 log units) of the biofilm after 20 min contact. In contrast, no such reduction in bacterial population was observed after contact for 6 h with the effective concentration of benzalkonium chloride for 5 min in suspension.

After the mechanical disruption of the biofilm, despite the presence of potentially interfering substances (matrix and cell components), and whatever the age of the biofilm, the bacteria were found to be as sensitive to the nine disinfectants as an equivalent population obtained on culture on TS agar and tested in suspension. Several workers have reported a reduction in the resistance of the bacteria after the mechanical disruption of the biofilm (Anwar et al, 1989; Evans et al, 1991; Johnston et Jones, 1995; Brown et al, 1995). Furthermore, dissociation of the biofilm with enzymes or surfactants has been found to enhance the bactericidal activity of a phenolic disinfectant (Jacquelin et al, 1994). The resistance of the bacteria included in a biofilm was thus not due to an alteration in metabolic state within the biofilm (slow growth and low metabolic activity).

The equivalent sensitivity we observed for the bacteria deposited on a surface to that of the bacteria included in biofilms was not in agreement with the observations of Maris (1992). This may be accounted for partly by differences in the method for preparation of the biofilms. The biofilms studied by Maris were obtained from a static 24-h-old culture on milk or calf serum (complex media rich in proteins), whereas our biofilms were obtained under dynamic conditions (flow culture) and with a medium containing much smaller amounts of potentially interfering substances. As the matrix retains substances from the medium, the richer the medium, the greater the interference from these substances in the biofilm will be. Under static conditions, live and dead bacteria may accumulate by sedimentation, which will also tend to increase the interfering action of the biofilm. Dubois-Brissonnet et al (1995) showed for *P aeruginosa* that biofilms obtained under static conditions were less sensitive to oxidizing agents (hypochlorite, peracetic acid/H_2O_2) than were biofilms produced under dynamic conditions with the same culture medium. Another explanation could be the difference in the quantity of the disinfectants used in our assays in the case of biofilms or carrier tests.

With our system (continuous culture flow reactor) even though the biofilm thickness increased with age, the population remained stable. This could be the result of an accumulation of dead or non-cultivable bacteria and/or exopolymers (Nickel et al, 1985; Zhang and Bishop, 1994). This accumulation could explain the increase of 10-day-old biofilm resistance compared to those of 2- and 5-day-old biofilm. The bacteria included in the 2- or 5-day-old biofilms had a similar resistance to that of the bacteria in the germ carrier tests. This is indicative of a small protective role by the matrix and a predominant influence of cellular adhesion (cell-support, cell-cell adhesion) or of cellular immobilization. LeChevalier et al

(1988b) have suggested that the physical obstruction exhibited by the surface to which the bacteria are attached hampers the access of the disinfectant to the cell membrane.

In conclusion, the biofilm matrix may hamper the penetration of antimicrobial agents into the biofilm via chemical, ionic and hydrophilic/hydrophobic interactions. However, the major factor in the resistance of biofilms to disinfectants appeared to be the reduced accessibility of the bacteria included in the biofilm. This could be attributed to a single factor or a combination of factors preventing access of the disinfectant to some parts of the cell membrane: i) adhesion to the support; ii) juxtaposition of cells; iii) immobilization by the matrix.

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