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ORIGINAL PAPER

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Comparative antibacterial potential of selected aldehyde-based biocides and surfactants against planktonic *Pseudomonas fluorescens*

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Abstract The antimicrobial efficacy of two aldehyde-based biocides (glutaraldehyde, GTA, and ortho-phthalaldehyde, OPA) and two surfactants (cetyltrimethyl ammonium bromide, CTAB, and sodium dodecyl sulphate, SDS) was tested against planktonic *Pseudomonas fluorescens*. The antimicrobial effects were evaluated by respiratory activity as a measure of the oxygen uptake rate, adenosine triphosphate (ATP) release, outer membrane proteins (OMP) expression and cellular colour changes. The results were compared with the bacterial characteristics without chemical treatment. Tests in the presence of bovine serum albumin (BSA), in order to mimic a disinfection process in the real situation under dirty conditions, were performed according to the European Standard EN-1276. *P. fluorescens* was completely inactivated with OPA (minimum bactericidal concentration, MBC = 0.5 mM) and CTAB (MBC = 5 mM) and was resistant to GTA and SDS. Only CTAB promoted cellular disruption and consequent ATP release. The antimicrobial action of the chemicals tested was significantly reduced when BSA was introduced into the bacterial cultures, increasing markedly the MBC values. Additionally, the presence of BSA acted as a disruption protective agent when CTAB was applied and stimulated the bacterial respiratory activity when lower concentrations of SDS were tested. The OMP of the bacterial cells was affected by the application of both surfactants. OMP expression remained unaltered after biocide treatment. Bacterial colour change was noticed after treatment with biocides and surfactants. In summary, *P. fluorescens* was extremely resistant to GTA and SDS, with antimicrobial action being quenched markedly by the reaction with BSA.

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Introduction

Control of microbial growth is required in many microbiologically sensitive environments where wet surfaces provide favourable conditions for proliferation of microorganisms [1, 5, 22]. The aim of a disinfection process is to reduce the viable microorganisms and to prevent microbial growth on the surfaces [31, 32]. According to Russell [26], there is an urgent need to investigate more fully the nature of the inhibitory and lethal effects of biocides and disinfectants. The array of possible cell multi-target sites would form an important aspect of such studies. According to the European Standard EN-1276 [8], the recommended procedure to evaluate chemical antimicrobial efficacy is based on tests using suspended cells.

Glutaraldehyde (GTA) and ortho-phthalaldehyde (OPA) are aldehydes that are known to have effective antimicrobial properties and are commonly used for high-level disinfection in healthcare environments [23, 27, 38]. GTA has been the reference product for disinfection for many years [23, 27, 37, 38]. This disinfectant has a broad spectrum of activity against bacteria and their spores, fungi and viruses, and a considerable amount of information is now available about the inactivation mechanisms [23]. OPA is a novel product that is claimed to have excellent microbiocidal, mycobactericidal and sporicidal activity [23, 27, 37]. These works indicate that OPA may also be used for microbial growth control in industrial systems. OPA received clearance by the FDA (Food and Drug Administration) in 1999 and is under study as an alternative to GTA for disinfection. OPA is an

aromatic compound with two aldehyde groups. It is suggested that the aromatic component permits OPA to penetrate the outer layers of cells, thus helping to explain the high activity against Gram-negative bacteria even although the degree of cross-linking is less than with GTA [23, 37]. Furthermore, OPA has several advantages compared to GTA: it is odourless, stable and effective over a wide pH range of 3–9, non-irritating to the eyes and nasal passages and does not require activation before use [27].

Surface active agents (surfactants) are a new class of agents with low–intermediate disinfectant potential that interact less with human tissues than the current aldehyde-based biocides. They are commonly used in mixtures of cleaning products because of their ability to lower the surface and interfacial tensions of liquids [3, 15]. These chemicals have the ability to wet surfaces, penetrate soil and solubilize fatty materials [3, 12]. Quaternary ammonium compounds (QACs) or cationic surfactants are often employed as disinfectants for manual processing lines and surfaces in the medical area, because of their excellent hard-surface cleaning, deodorization and antimicrobial properties [21, 23]. The mode of action is attributed to the positive charge, which forms an electrostatic bond with negatively charged sites on microbial cell walls [23]. Those electrostatic bonds create stress in the cell wall, leading to cell lysis and death. QACs also cause cell death by protein denaturation, disruption of cell wall permeability and reduction of the normal intake of nutrients into the cell [1, 4]. Cetyltrimethyl ammonium bromide (CTAB) is a QAC that is considered to rupture the cell membrane. The primary site of action of CTAB has been suggested to be the lipid components of the membrane causing cell lysis as a secondary effect [11]. Anionic surfactants possess strong detergent but weak antimicrobial properties, except at high concentrations, when they induce lyses of Gram-negative bacteria [14]. The outer and cytoplasmic membranes and the membrane-bound enzyme environment are the main targets [7]. For example, sodium dodecyl sulphate (SDS) is an anionic surfactant widely used in detergent formulations [15].

The aim of this work is to clarify the antimicrobial potential and mode of action of two aldehyde-based biocides and two surfactants against planktonic *P. fluorescens*. Further studies were performed with a model protein in order to mimic dirty conditions found in real situations, according to the European Standard EN-1276 [8].

Materials and methods

Chemicals tested

In the present work, the following chemical agents were used.

Two non-oxidizing aldehyde-based biocides

- Glutaraldehyde that was purchased from Riedel-de-Haën (Cat. No. 62621) and the concentrations tested were 2, 4, 8, 15, 20, 35, 50, 70, 100 and 130 mM.
- Ortho-phthalaldehyde that was purchased from Sigma (Cat. No. P-1378) and the concentrations tested were 0.02, 0.04, 0.1, 0.2, 0.25, 0.35, 0.4, 0.5, 0.75, 1.5 and 2 mM.

Two surfactants

- Cetyltrimethyl ammonium bromide, a cationic surfactant, was purchased from Merck (critical micellar concentration, CMC: 1.00 mM; Cat. No. 102342). The concentrations tested were 0.125, 0.250, 0.500 and 0.900 mM.
- Sodium dodecyl sulphate, an anionic surfactant, was purchased from Riedel-de-Haën (CMC: 8.30 mM; Cat. No. 62862). The concentrations tested were 0.5, 1, 3 and 7 mM.

The concentrations tested were based on previous studies performed with *P. fluorescens* biofilms [31].

Microorganism and culture conditions

Pseudomonas fluorescens ATCC 13525^{T} was the microorganism used throughout this study. A continuous pure culture of *P. fluorescens* was grown in a 2-l glass chemostat, at 27°C, aerated (air flow rate = 0.425 min^{-1}) and agitated with a magnetic stirrer (Heidolph Mr 3001) in order to have microorganisms in the exponential phase of growth. The chemostat was continuously fed with 40 ml/h of sterile medium containing 5 g/l glucose, 2.5 g/l peptone and 1.25 g/l yeast extract in 0.02 M phosphate buffer (KH_2PO_4; Na_2HPO_4), pH 7.0.

Disinfection procedure

A suitable volume of the bacterial culture was harvested from the 2-l reactor, washed with saline (0.85%-w/v NaCl) phosphate buffer (0.02 M) by three consecutive steps of centrifugation (3,777*g*, 5 min) and resuspended in 0.02 M phosphate buffer, pH 7, in order to obtain a suspension with an optical density (λ = 640 nm) of 0.4 (bacterial cell density of approximately 1×10^{9} cells/ml). This bacterial culture was then divided by several sterilized glass flasks, put in an orbital shaker (120 min^{-1}, 27°C) and exposed to different concentrations of the various chemicals for 30 min.

The effect of the chemicals was assessed by determining the bacterial respiratory activity through oxygen consumption, the assessment of the adenosine triphosphate (ATP) release and the analysis of the outer membrane proteins (OMP) expression. Before the

assessment of the bacterial respiratory activity the antimicrobial agents were chemically neutralized.

To investigate the influence of proteins on the antimicrobial efficacy, the procedure described above was followed but with the previous addition of 3 g/l of bovine serum albumin (BSA) (Merck 12018) to the bacterial suspension, simulating, by this way, dirty conditions [8].

Disinfection procedures were performed at least three times for each conditions tested.

Neutralization of the chemicals

After the contact of the chemical agent with the cells, they were subjected to a process of neutralization in order to quench their antimicrobial activity, according to Johnston et al. [17]. The aldehyde-based biocides were neutralized with sodium bisulphite (Sigma) at a final concentration of 0.5% (w/v). The surfactants were chemically neutralized by the following solution: (w/v) 0.1% peptone, 0.5% Tween 80 (Sigma) and 0.07% lecithin (Sigma), dissolved in phosphate buffer, pH 7. A concentrated neutralization solution was prepared and autoclaved prior to utilization.

Respiratory activity assessment

The respiratory activity (respirometry) was determined by measuring oxygen uptake rates in a Yellow Springs Instrument (OH, USA): biological oxygen monitor (BOM; Model 53) and the procedure used has been described elsewhere [32]. The samples were placed in the temperature-controlled vessel of the BOM ($T = 27 \pm 1$°C). Each one contains a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for 30 min to ensure oxygen saturation ($[O_2] = 9.2$ mg/l). The vessel was closed and the decrease of oxygen concentration was monitored over time. The initial linear decrease observed corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, 50 µl of a glucose solution (100 mg/l) was added in each vessel. The slope of the initial linear decrease in the DO concentration, after glucose injection, corresponds to the total respiration rate. The difference between the two respiration rates gives the oxygen uptake rate due to glucose oxidation.

The minimum bactericidal concentration (MBC) for each method was determined as the lowest concentration of the chemical agent where no respiratory activity was detected, according to Johnson et al. [16].

ATP measurement

The ATP released from the cells was measured with the luciferase–luciferine System /Sigma FL-AAM. After the required contact time with the chemicals, 100 µl of the cellular suspension was added to 100 µl of a 25-fold dilution mixture of luciferine and luciferase. The light transmission was measured in a bioluminometer (Lumac, Biocounter M 25000) and the output values were recorded in relative light units (RLU). In order to investigate possible interference of the chemicals with the bioluminescent method and with the ATP stability, control experiments were carried out with phosphate buffer in the presence and absence of chemicals and the respective neutralizing agents. Control experiments in the presence of BSA were also performed with CTAB. The effect of the chemical agents on the bacterial integrity was evaluated in terms of RLU as an estimative of the intracellular ATP content released. The latter was calculated, according to Dalzell and Christofi [6], using the equation below:

$$\text{relative light units} = (\text{RLU}_1/\text{RLU}_0) \quad (1)$$

where RLU_0 is the RLU of the control assay (bacteria without chemical addition) and RLU_1 is the RLU of the test sample.

Culture biomass quantification

The dry biological mass (cells) was assessed by the determination of the total volatile solids according to the APHA, AWWA, WPCF Standard Methods, method number 2490 A-D [2].

OMP isolation

The OMP profiles of cells without treatment and after treatment for 30 min with antimicrobial representative concentrations (2 mM of GTA, 0.5 mM OPA, 0.9 mM CTAB and 7 mM of SDS) were analysed using SDS-polyacrylamide gel electrophoresis (PAGE). The OMP was isolated according to the method described by Winder et al. [40]. The cells were harvested by centrifugation (3,777*g*, 5 min, 4°C). The pellet was suspended in 25 mM Tris and 1 mM $MgCl_2$ buffer (pH = 7.4). The bacterial suspension was sonicated for 2 min (Vibracell, 60 W) on ice to promote cell lysis. After sonication the solution was centrifuged (7,000*g*, 10 min, 4°C) in order to remove non-lysed cells. The supernatant was collected and N-lauroylsarcosine was added to obtain a final concentration of 2% (w/v), in order to solubilize the inner membrane proteins. This solution was left on ice for 30 min. Afterwards, the solution was centrifuged (17,000*g*, 1 h, 4°C) to recover the OMP. The pellet containing the OMP was resuspended in 1 ml of deionized water and stored at −20°C until required.

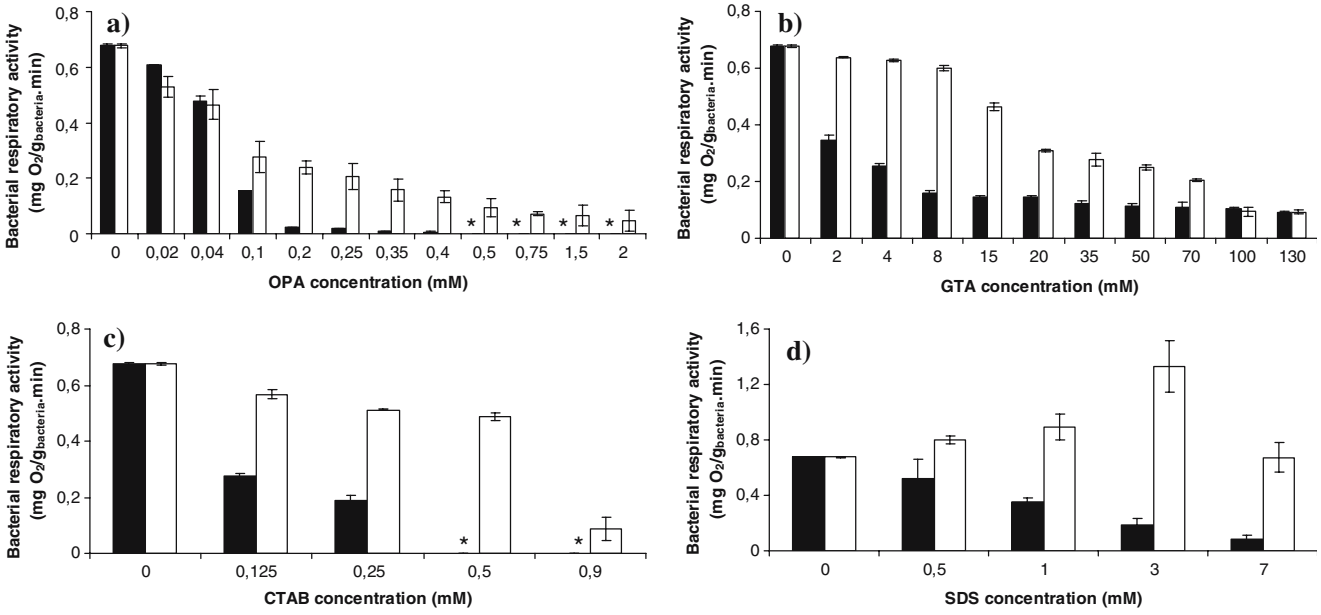

Fig. 1 Respiratory activity of the bacterial suspended cultures, after treatment with several concentrations of OPA (**a**), GTA (**b**), CTAB (**c**) and SDS (**d**), with (*white bars*) and without (*black bars*) BSA addition. *Each symbol* indicates the mean ± SD of several independent experiments. The *asterisks* indicate that the bacteria are totally inactivated

OMP analysis

The protein content of the OMP samples was determined using the Bicinchoninic Acid Protein Assay Kit (BCA) (BCA–PIERCE Cat. No. 23225) with BSA as standard. This procedure was applied in order to insert in the gel cassettes the same OMP concentration for the several samples (240 ± 10 μg/ml).

The OMP samples obtained were subjected to SDS-PAGE, as reported by Laemmli [19] with 12% (w/v) acrylamide. Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the gels were Coomassie blue and silver stained for protein profile detection. Thereafter, the gels were scanned using a GS-800 calibrated densiometer and analysed by the Quantity One 4.5.2 software (BioRad).

Cellular colour changes

The colour of each bacterial pellet, after chemical treatment, obtained during the OMP extraction procedure was recorded (Camera Sony Digital Mavica 10 ×) and compared with the starting colour of the bacteria without chemical treatment.

Statistical analysis

The Student's *t* test was performed when the aim was to investigate whether the differences between the experimental values obtained under different conditions could be considered significant.

Results

Respiratory activity after exposure to the chemicals

The effect of OPA, GTA, CTAB and SDS on the respiratory activity and consequent viability of *P. fluorescens* in the presence and absence of BSA is presented in Fig. 1.

From Fig. 1a, it can be seen that an increase in OPA concentration promoted a decrease in the respiratory activity, causing inactivation. For a concentration equal and higher than 0.5 mM total bacterial inactivation was achieved (MBC = 0.5 mM). However, the antimicrobial action of OPA was severely affected in the presence of BSA (MBC > 2 mM), this reduction being particularly noticeable for OPA concentrations higher than 0.1 mM. In the presence of BSA, total inactivation of the bacterial suspension was not observed even when high OPA concentrations were used. Statistical analysis of the results obtained in the presence and absence of BSA showed that they were significantly different ($P < 0.01$).

The results of the bacterial respiratory activity after GTA application show (Fig. 1b) that this biocide was not effective in the total inactivation of the cells (MBC > 130 mM). Even after the treatment with 130 mM of GTA the cells still had respiratory activity. However, an increase in the GTA concentration promoted a decrease in the respiratory activity. Concerning the interference of BSA with the antimicrobial action of GTA, the presence of BSA reduced the GTA antimicrobial potential. Statistical analysis reveals that the results with and without BSA presence are significantly different ($P < 0.05$).

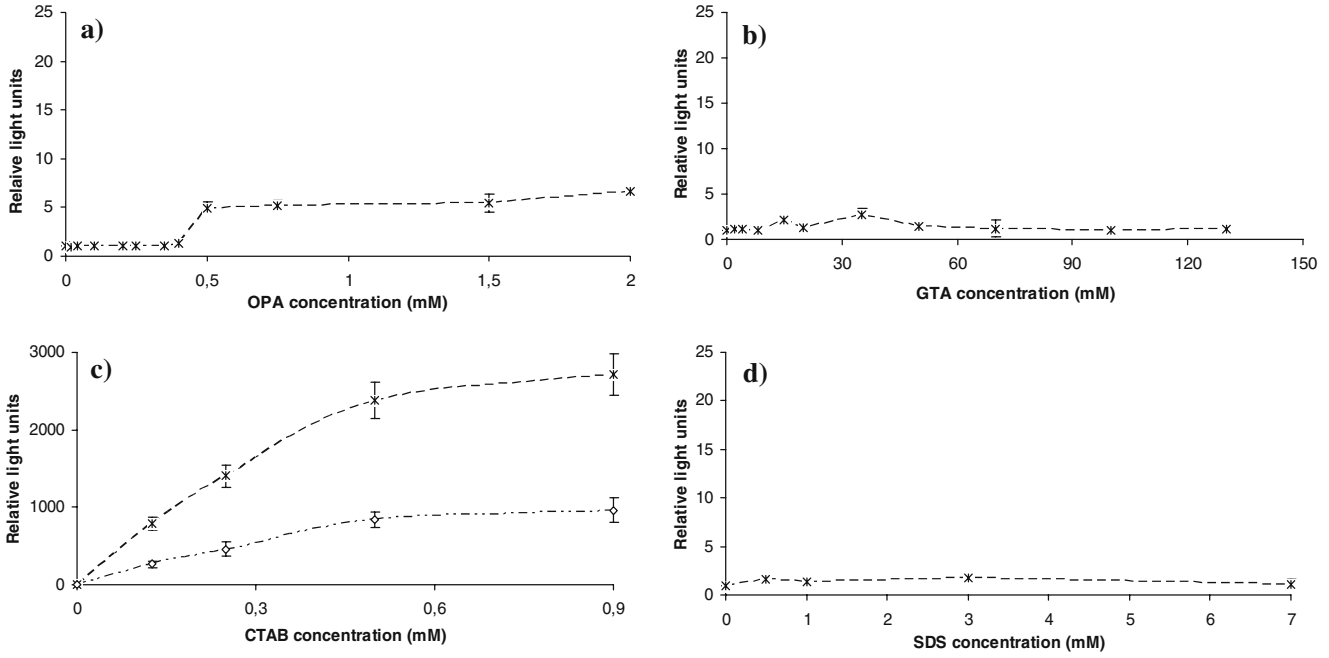

Fig. 2 Relative light units as a measure of the ATP released from the bacterial cells after treatment with several concentrations of OPA (**a**), GTA (**b**), CTAB—in the presence (*diamond*) and absence (*asterisks*) of 3 g/l of BSA (**c**) and SDS (**d**). *Each symbol* indicates the mean ± SD of several independent experiments

P. fluorescens respiratory activity decreases with the CTAB application (Fig. 1c), this decrease being a function of the CTAB concentration increase. Total bacterial inactivation was achieved for a concentration of 0.5 mM (MBC = 0.5 mM). The presence of BSA reduced significantly ($P < 0.05$) the antibacterial efficacy of CTAB (MBC > 0.9 mM). Concerning the effect of SDS on the bacterial respiratory activity, Fig. 1d showed that SDS promotes significant reduction on the bacterial respiratory activity. Nevertheless, with the range of concentrations tested, SDS did not promote the total inactivation of *P. fluorescens* (MBC > 7 mM). Figure 1d also shows that in the presence of BSA the bacterial respiratory activity increased reaching values even higher than the ones observed for the control experiment. This fact suggests that the antimicrobial effect of SDS was quenched and respiratory activity stimulated. It seems that there was an optimal SDS/BSA rate that promoted surfactant neutralization and cellular activity induction. For instance, with the application of 3 mM of SDS and in the presence of BSA, the bacterial respiratory activity is approximately two times higher than the one obtained without SDS application. The statistical analysis of the results, obtained in the presence and absence of BSA, shows that they are significantly different ($P < 0.05$).

ATP release after exposure to the chemicals

Bioluminescence assays to determine ATP release were performed as an attempt to ascertain if the antimicrobial action of the several chemicals could have an effect on the bacterial integrity (Fig. 2).

The ATP bioluminescence assay showed that the RLU detected in the bacterial suspensions after OPA, GTA and SDS treatment (Fig. 2a, b, d) are insignificant and similar to the RLU observed without chemical addition, meaning that cellular disruption did not occur after exposure to the referred chemicals. Regarding the bioluminescence results after CTAB treatment, the ATP released into the medium increased with CTAB concentration (Fig. 2c), suggesting that this surfactant promotes cellular disruption to an extent dependent on the CTAB doses. An additional ATP bioluminescence experiment was performed, but in the presence of BSA, showing that, RLU are always lower when compared without the BSA presence, regardless of the CTAB concentration applied. This observation is clearly a sign of the BSA ability to protect the bacterial cells. This fact helps to understand why, even with CTAB concentrations higher than 0.5 mM, total bacterial inactivation was not achieved. So, the presence of BSA has a protective effect against CTAB action, leading to the release of a lower ATP concentration ($P < 0.05$). Comparing the CTAB results obtained by respirometry (Fig. 1c) and the bioluminescent methods (Fig. 2c), a strong relationship ($R^2 = 0.975$) was found between bacterial inactivation and RLU, meaning that an increase in bacterial inactivation corresponds to a proportional increase in the ATP released by the cells ($P > 0.1$). Concerning the same data but in the presence of BSA, the correlation coefficient found, $R^2 = 0.602$, was undoubtedly poor. This latter relationship may indicate

that when BSA was present in the suspension, bacterial inactivation was related to the ATP released and probably the presence of BSA acted as a protective agent to the cells avoiding their disruption, as presented in Fig. 2c.

Effect of the chemicals on OMP expression

The OMP profiles of *P. fluorescens* were assessed after treatment with representative chemical concentrations (2 mM of GTA, 0.5 mM of OPA, 0.9 mM of CTAB and 7 mM of SDS) in order to assess the effect of several chemical agents on OMP expression and are presented in Fig. 3.

The tested concentrations of the aldehyde-based biocides did not cause under-expression of the major OMP (18 ± 3, 22 ± 3, 32 ± 2, 34 ± 2 kDa) when contrasted with the experiment without chemical treatment (Fig. 3). CTAB seems to promote a dilution in the OMP content presented in the electrophoresis gel after staining and the over-expression of an OMP with an apparent molecular weight of 50 ± 2 kDa. It is interesting to note that the molecular weight of the over-expressed CTAB induced OMP is similar to the molecular weight of the well-known OprM protein responsible for multiple drug resistance on *Pseudomonas* spp. [20]. The OMP expression after CTAB treatment corroborates the result of the ATP measurement, because the existence of ATP release requires the destabilization of the outer membrane, and probably changes in the proteins involved in the efflux phenomenon. The effect of SDS on the cells resulted in the increase of OMP bands with higher molecular weights, maintaining the major OMP.

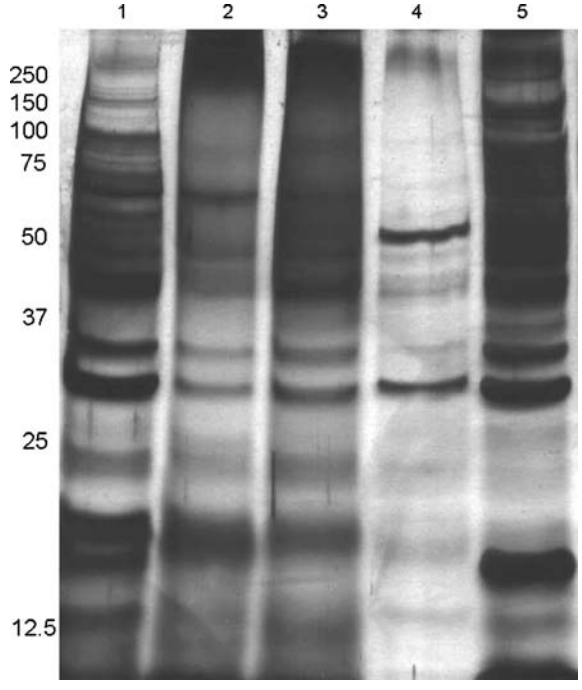

Fig. 3 Outer membrane proteins profile of *P. fluorescens* cells without chemical treatment (*lane 1*) and after treatment with GTA (*lane 2*), OPA (*lane 3*), CTAB (*lane 4*) and SDS (*lane 5*). *Numbers* on the left represent molecular weights in kDa

Cellular colour changes

Analysis of the cellular colour before and after treatment with representative concentrations of the tested chemicals demonstrated that different chemical agents interacted with the bacterial cells promoting colour changes. Before exposure to the chemical agents, the pellet of cells looked like pink, while after treatment with 2 mM of GTA they look red/pink; with 0.5 mM of OPA they look dark green; with 0.9 mM of CTAB they look white/cream; and with 7 mM of SDS they look white.

Discussion

Biocides and surfactants are chemical agents with antimicrobial properties revealed by a multiplicity of biochemical targets [14, 22]. These features make difficult the analyses of the mechanism of action which is reflected by the low number of reports concerning this subject. The improvement in the understanding of the relationships between biocide/surfactant molecular properties, antibacterial potential and mechanisms of action could facilitate the design of chemical mixtures that more effectively control microbial proliferation under different environmental conditions. An effective and wide spectrum disinfection strategy helps to overcome the problems of cross-resistance, existence of persistent populations or the formation of recalcitrant and multiresistant biofilms in disinfection dependent processes [22, 29, 30]. *P. fluorescens* was used as a model microorganism since it is ubiquitous and has a potential to cause serious problems in industrial environments in its planktonic and biofilm states [29, 30, 32, 39]. This bacterium has a strong ability to form disinfectant resistant biofilms [29, 30]. Simões et al. [29] verified the failure of OPA to effectively inactivate and remove *P. fluorescens* biofilms adhered to stainless steel surfaces. This phenomenon was attributed to the protective effect provided by the extracellular polymeric matrix in biofilms, mainly composed of proteins and polysaccharides [29].

Short-term respiratory activity was used to assess the antimicrobial efficacy of the chemicals tested. This methodology was demonstrated previously to be rapid, reliable, economic and easy to use against aerobic, heterotrophic bacteria [32]. Additionally, specific methods based on the physiological or metabolic activity supply more information on antimicrobial agents than the ability to grow and form colonies on a solid media [35]. The traditional methods of bacterial enumeration by colony formation on agar may overestimate antimicrobial efficacy since bacteria may remain viable after chemical

application but may not grow on solid media. This loss of culturability led to the assumption that bacterial cells in this state were viable but not culturable [32]. Those cells lose their ability to form colonies, but they remain physically intact and metabolically active, allowing the further appearance of persistent cells [22, 32].

The selection of a suitable chemical agent plays a significant role in disinfection. Some of the chemical agents tested in the present study are known to be used as disinfectants and in disinfectant formulations [13, 27]. Indeed, all the chemical agents tested reduced the bacterial respiratory activity. Comparing the antimicrobial efficacy within related chemical nature agents, the results demonstrated that OPA is more efficient in the inactivation of suspended cells than GTA. GTA was used as a reference chemical due to the considerable amount of information available concerning its antimicrobial potential [23, 27, 33, 37, 38]. The MBC for OPA was 0.5 mM (Fig. 1a), whereas for GTA even with a concentration of 130 mM there was no total reduction on the respiratory activity (Fig. 1b). This latter evidence reinforces that OPA is a good alternative to GTA for disinfection, as already suggested in several studies [13, 37]. The mechanism of antimicrobial action of both the aldehyde-based biocides is related to the characteristics of the chemicals, since GTA and OPA are known to stabilize the outer membrane and cell walls of vegetative bacteria by blocking the passage of essential nutrients to the cell [27, 37]. Both biocides were already known to increase biofilm mechanical stability due to increased cellular cross-linking and the consequent difficulty in removal from the surface [30, 31]. The bactericidal effect of OPA is attributed to its lipophilic aromatic nature that facilitates passage through the outer layers of Gram-negative bacteria [33]. The OPA and GTA efficacy were considerably reduced when BSA was introduced in the suspended bacteria (Fig. 1a, b), emphasizing that an effective cleaning regime must be performed before the application of disinfectants. The reasons for the reduction are probably related to its capacity to act as a neutralizer of GTA and OPA, hence lowering the amount of biocide available to react with the cells [27, 37]. The formation of a BSA layer which coated the cells can account for the reduction of the bactericidal efficacy of both biocides when the protein was present in the cultures [9]. Additionally, it is known that BSA contains histidine and glycine and they can neutralize GTA and OPA, respectively, making them safe for disposal [27]. In fact, the interfering substances shall be chosen according to the specific application of the chemical product [8]. According to the European Standard EN-1276 [8], in addition to BSA, other interfering substances can be used: milk (dairy-associated industry); yeast extract (breweries); sucrose (beverage, soft drink industries), pH 5.0 and pH 9.0 buffer solutions (cleaning in place); sodium lauryl sulphate (cosmetic area).

The chemical interaction between the aldehyde-based biocides and proteins is reinforced by the bacterial colour changes after chemical treatment. These results are in accordance with the ones obtained by Walsh et al. [37], proposing that this effect is a consequence of the reaction with amino acids. A red colour developed after treatment with GTA resulted from an interaction of the dialdehyde with non-peptidoglycan components in the outer layers of Gram-negative bacteria [33]. However, this reactivity of OPA and GTA with proteins does not affect the proteins of the outer membrane or does affect them to a small extent that is not detected in SDS-PAGE analysis (Fig. 3). The molecular weights of the major OMP expressed are similar to the molecular weights of the major OMP characteristics of *Pseudomonas* spp.—OprH or OprL (18 ± 3 kDa); OprG (22 ± 3 kDa); OprF (32 ± 2 kDa); OprE (34 ± 2) [18].

Regarding the comparison of both surfactants, the anionic and cationic nature influences the antimicrobial properties of the surfactants. This feature played a significant role in biofilm control, where the strength of the electrostatic interactions between CTAB and *P. fluorescens* biofilms promoted the decrease in mechanical stability. On the other hand, SDS increased the biofilm mechanical stability and was consequently more difficult to remove from the surfaces [31]. In the present study, CTAB at 0.5 mM promoted total bacterial inactivation, a concentration half of the CMC. However, BSA decreased bacterial inactivation (Fig. 1c). This fact demonstrates the strong interaction between CTAB and proteins. This surfactant also promoted the release of intracellular ATP, this release being to a small extent in the presence of BSA, probably, due to its protective effects on the bacterial cells (Fig. 2c). The bacterial inactivation and ATP released into the medium, after CTAB aggression, were concentration dependent (Fig. 2c), increasing with surfactant concentration. Furthermore, CTAB promoted a dilution in the OMP content and the apparent (immunoblot analysis are under-running) overexpression of the OprM—50 ± 2 kDa OMP (Fig. 3). This OMP (OprM) is known to be involved in multiple drug resistant systems [20]. One of the important roles of the bacterial membrane transport systems is the provision of resistance to antimicrobial agents. Efflux mediated resistance has been shown to include a variety of structurally unrelated antimicrobial agents including antibiotics and biocides [22, 38]. The OMP results corroborate the amount of ATP verified after CTAB treatment since the existence of ATP release required the destabilization of the outer membrane. Moreover, CTAB is a known chemical used for microbial cell lysis and is routinely used for this purpose in DNA analysis methods [28]. Thus, missing protein bands in the OMP profile (Fig. 3) could be, arguably, a result of the partially damaged cellular outer envelope, leading to the loss of OMP rather than the impaired biosynthesis of certain OMP. According to several reports [1, 25], cationic surfactants have a general antimicrobial action, leading to the disruption of the cell membranes due to protein interaction, consequently to the depolarization of the cytoplasmic membrane and the leakage of the

cytosol components. Ultrastructural changes may be induced by the action of the cationic surfactants, producing dramatic effects on the bacterial envelopes and causing lysis or massive leakage of the cell components.

Concerning the application of SDS, total inactivation was not achieved (Fig. 1d). Moreover, SDS interacted strongly with BSA allowing for the increase of respiratory activity for the lower concentrations tested (0.5 and 1 mM), an effect probably related to the chemical neutralization of SDS by BSA. Hydrophobic interactions are behind the reaction of surfactants with BSA in a controlled chemical system [10]. Additionally, several authors have reported that *Pseudomonas* spp. could biodegrade SDS and incorporate the hydrophobic metabolites of the alkyl chain as cellular components, such as membrane lipids. In fact, the bacteria/BSA/surfactant system resulted in an enhanced glucose uptake, stimulating the respiratory activity and resulting in a SDS–glucose mediated uptake pathway [34, 36]. This result emphasizes the care needed in the correct choice of a chemical agent for disinfection. Moreover, OMP changes could be an indication of bacterial adaptation and resistance to the chemical agents since the application of SDS resulted in the increase of OMP bands with higher molecular weights (Fig. 3). Several authors proposed that surfactants, due to their detergent properties, react strongly with the cell outer membrane, particularly the OMP [4, 7, 12]. Paulus [24] also pointed out that the antimicrobial effect of anionic surfactants is restricted mainly to Gram-positive bacteria and that their point of attack is apparently the microbial cell membrane.

The interaction between the surfactants tested and the bacterial cells resulted in colour changes, a phenomenon arguably related with OMP reaction. In fact, the results suggest that the mechanisms of resistance of *P. fluorescens* when exposed to the tested surfactants may involve changes in some OMP, as differences were observed in the SDS-PAGE experiments.

In conclusion, *P. fluorescens* was extremely resistant to GTA and SDS, the antimicrobial action of the tested chemicals being quenched markedly by the reaction with BSA. This fact proposes that the processes of disinfection under dirty conditions need the application of improved methods in order to avoid the effect of interfering substances that react with the antimicrobial agents, lowering, by this way, the amount available to react with the cells. The presence of BSA promoted the decrease in the antimicrobial efficacy of every chemical agent tested. Moreover, in the case of CTAB, BSA also acted as a cell protective agent since the ATP release was significantly impaired. In the case of SDS, the presence of proteins even enhanced the bacterial respiratory activity when lower concentrations of SDS were applied. The OMP of the bacterial cells is affected by the application of both surfactants. Even though aldehyde-based biocides have the ability to react with proteins, the OMP profile was not affected after treatment with GTA and OPA. The several antimicrobial agents tested promoted bacterial colour changes, a phenomenon arguably related with protein interactions.

This preliminary study suggests that further analyses are required in order to ascertain the molecular basis (by proteomic studies) of the increased resistance of *P. fluorescens* when exposed to some biocides and surfactants, such as GTA and SDS, and to assess the synergistic effect of biocides and surfactants.

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