Enterobacter gergoviae membrane modifications are involved in the adaptive response to preservatives used in cosmetic industry

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Abstract

Aim: The objective of this study was to understand the adaptive mechanisms in *Enterobacter gergoviae* which are involved in recurrent contaminations in cosmetic products that are incorporated with preservatives.

Methods and Results: Bacterial strains from two backgrounds were examined for a profound understanding of the mechanisms of adaptation against preservatives. It included a series of *Ent. gergoviae* strain—ATCC 33028 derivatives, isolated using increasing methylisothiazolinone–chloromethylisothiazolinone (MIT-CMIT) and triclosan concentrations. The other series was of *Ent. gergoviae* isolates from cosmetic products exhibiting MIT-CMIT and triclosan resistance. We evaluated the outer membrane protein modifications and efflux mechanisms activities responsible for the resistant trait via immunoblotting assays. Additionally, for understanding the efflux activity real-time efflux, experiments were performed. A cross-insusceptibility between preservatives and some disinfectants was observed in MIT-CMIT-resistant derivative isolates, but antibiotics susceptibility was not altered. Resistance to EDTA was significant in all preservatives insusceptible derivative strains, indicating modifications in the LPS layer. Furthermore, an array of real-time efflux assays indicated different activity levels while no variations were detected in porins and AcrAB-TolC pumps production. Overexpression of a specific flagellin-type protein was observed in one of the MIT-CMIT- and triclosan-resistant strains. Another candidate, a 25-kDa peroxiredoxin enzyme involved in oxidative detoxification, was identified to be overexpressed in MIT-CMIT derivative. A similar profile was also observed among strains isolated from cosmetic products.

Conclusions: Our study highlights the existence of adaptive mechanisms such as overexpression of detoxifying enzymes, flagellin, modification of membrane structure/function in *Ent. gergoviae*. They might be involved in recurrent episodes of contaminations occurring in the cosmetic production lines.

Significance and Impact of the Study: No cross-resistance could be observed with antibiotics when MICs to preservatives were increased; however, a decrease in the disinfectants bactericidal effects was confirmed in preservative-tolerant strains. This will impact industry disinfection strategies treatment against bacteria.

Introduction

Enterobacter gergoviae is a Gram-negative bacteria often identified in awry manufactured or spoiled cosmetic products (Anelich and Korsten 1996; Davin-Regli *et al.* 2006). Recently, we have confirmed the genetic diversity of strains involved in cosmetic contaminations and the bacteriostatic action of preservatives which results in increased bacterial insusceptibility and thus its emergence (Périamé *et al.* 2014). These days, to ensure microbiological quality, consumer safety and the organoleptic properties of cosmetic products, manufacturers need to comply with defined standards (BIO ECOCERT, COSMOS) (Hiom 2013). As a result, cosmetic production requires disinfection steps in the production chain before and during the manufacture/conditioning stages. In this disinfection process, two parameters, concentration and contact time of preservative, play a key role, for *example* in case of methylisothiazolinone–chloromethylisothiazolinone (MIT-CMIT) (Maillard 2013; Maillard *et al.* 2013). A drawback regarding the usage of these preservatives is the possibility to generate cross-insusceptibility to other disinfectants or preservatives among the microflora (Maillard 2013; Maillard *et al.* 2013). Hence, understanding the molecular mechanism involved in the rise of this adaptation is necessary. This will lead to identification of novel drug targets and hence promote new modes of counteracting them. To attain this understanding, we have examined insusceptible strains derivatives from the susceptible *Ent. gergoviae* ATCC 33028, using increasing MIT-CMIT and triclosan concentrations (Rensch *et al.* 2013). These compounds are used in cosmetic industry for disinfection process. The resulting derivatives exhibited insusceptibility to biguanides and nonionic surfactants-amines which have a common mode of action on bacterial membranes (Broxton *et al.* 1984; Ikeda *et al.* 1984; Allen *et al.* 2006; Ortega Morente *et al.* 2013). The characteristics of these adapted strains were studied to determine the involvement of common mechanisms active in preservatives and in biocides insusceptibility. This point is of special importance with the European commission regulation concerning the use of biocidal products currently used in cosmetic formulations and the necessity to ensure that, when properly used, they are sufficiently effective and have no unacceptable effect on the target organisms such as resistance (Anon 2012).

Materials and methods

Bacterial strains, derivative strains and growth conditions

Enterobacter gergoviae clinical strain ATCC 33028, its biocide-resistant derivatives and three strains, namely eg23, eg25 and eg26, isolated from contaminated products from a cosmetic company (Périamé *et al.* 2014) were used (Table 1). These three cosmetic strains were chosen for various preservatives susceptibility tests, out of a panel of 47 *Ent. gergoviae* isolated from cosmetic products (Périamé *et al.* 2014). Bacteria were grown at 37°C in Luria–Bertani (LB) broth (Difco Laboratories, Detroit, MI, USA). Insusceptible derivate strains were successively obtained from ATCC 33028, which were grown in the presence of methylisothiazolinone, MIT-CMIT or triclosan, using culture with increased stepwise concentrations ranging from 1/2 MIC to 6 × MIC of the selected compounds. Four MIT-CMIT insusceptible derivatives were obtained at $1{\cdot}875{\cdot}10^{-4}$%, $3{\cdot}75{\cdot}10^{-4}$%, $4{\cdot}5{\cdot}10^{-4}$% and $6{\cdot}10^{-4}$% concentrations and named M1, M2, M3 and M4, respectively. Similarly, three triclosan insusceptible derivatives were obtained at 5, 10 and 20 g l^{-1} concentrations, which were named T1, T2 and T3, respectively (Table 1). Mueller–Hinton broth (MHB) was used as the bacterial medium for preservatives, antibiotics and chemicals susceptibility tests.

Disinfectants susceptibility, MIC and MBC determination

Susceptibility to disinfectants was tested for peroxyacid (P3-oxonia), aminopropyl dodecylpropane-diamine combined with polyhexamethylene biguanide (ADD/PHMB) Bactanios2000 and laurylamine dipropylenediamine (Aniosteril DAC II). These actives are currently used in cosmetic industry during the production steps. The minimum inhibitory concentrations (MICs) were determined by the microdilution method (CLSI) in MHB in microplates as previously described (Kuete *et al.* 2011). MBCs were determined using Eugon LT100 broth in 96-well plates, which is commonly used in cosmetic industry for the enrichment of aerobic bacteria in cosmetic products and preservatives neutralization according to ISO standards (Quisno *et al.* 1946).

Table 1 Bacterial *Enterobacter gergoviae* strains and corresponding derivative-resistant strains used in this study

Origin	Name in this study
Cosmetic Industry (Périamé *et al.* 2014)	eg23
Cosmetic Industry (Périamé *et al.* 2014)	eg25
Cosmetic Industry (Périamé *et al.* 2014)	eg26
Ent. gergoviae, ATCC 33028 selected with MIT-CMIT (at $1{\cdot}875{\cdot}10^{-4}$%, $3{\cdot}75{\cdot}10^{-4}$%, $4{\cdot}5{\cdot}10^{-4}$% and $6{\cdot}10^{-4}$%, respectively)	M1, M2, M3, M4
Ent. gergoviae, ATCC 33028 selected with triclosan (at 5, 10 and 20 μg ml^{-1}, respectively)	T1, T2, T3

The medium is composed of a mixture of peptones, cystine, glucose and salts which favour the growth of a wide variety of micro-organisms. Lecithin and polysorbate 80 neutralize the antibacterial activity of most antiseptics or preservatives (Williamson and Kligman 1965).

Briefly, after MIC determination in MHB, the bacterial wells showing growth inhibition were resuspended at 1/10 and 1/100 in Eugon LT100 broth and re-cultivated. The MBC was defined as the lowest bactericidal concentration of preservatives required to kill bacteria after incubation at 30°C for one to 4 days as recommended for the enrichment of aerobic bacteria in cosmetic products according to ISO standards, respectively (Anon 2009). The determinations were carried out in independent triplicates.

Antibiotics, preservatives and chemical susceptibility testing by MIC determination

Cefepime (CEF), nalidixic acid (NAL), polymyxin B (POL), ceftazidime (CAZ) and imipenem (IMI) were used to assess the antibiotic susceptibility of the various strains. Triclosan and MIT-CMIT were used to assess derivatives adaptation to these biocides compared to the reference strain and those isolated from cosmetic formulations. Antibiotics were prepared and used according to the microdilution method in MHB as previously described for disinfectants. Phenylalanine-arginine β-naphthylamide (PAßN) used at low concentrations has been previously reported to block the *Enterobacteriaceae* efflux pump such as AcrAB-TolC and restore the activity of antibiotic molecules that are well described as efflux pump substrates (Mamelli *et al.* 2009; Kuete *et al.* 2011). PAßN was thus used as efflux pump inhibitor on the various *Ent. gergoviae* strains. The inoculated microplates were incubated at 30°C for 18–24 h. An efflux pump activity was identified when the PAβN addition induced a threefold decrease in MIC value for an antibiotic molecule (Ghisalberti *et al.* 2005). Each assay was repeated independently three times, and the MIC values corresponded to the mean of these triplicates.

A way to circumvent alteration of bacterial membrane barrier in resistant strains is to use chaotropic agents or detergents (Mamelli *et al.* 2009; Bolla *et al.* 2011). MICs determination of a chelating agent such as EDTA, which is used as a common food preservative or a detergent such as sodium deoxycholate (DOC), were employed to investigate bacterial outer membrane integrity of ATCC 33028 strain as compared to triclosan and MIT-CMIT derivative-resistant strains. The MIC assays were performed in 96-well plates. EDTA was prepared using a twofold dilution method in MHB. Wells containing 100 μl of EDTA dilution (2×) were inoculated with 100 μl of cell suspension prepared by diluting a culture in double strength MHB (2×) to obtain viable counts of approximately 10^5 CFU ml^{-1}. For combination assays, EDTA was used at 1 mmol final concentration as previously reported (Mamelli *et al.* 2009).

β-Lactamase assays

The various bacterial cultures were incubated overnight in the presence of imipenem (at subinhibitory concentration) to induce the expression of ß-lactamase (Fadli *et al.* 2012) with the respective concentrations of preservatives (MIT-CMIT and triclosan) corresponding to the different derivatives strains. Cells were resuspended in fresh medium in the presence of imipenem and washed (when OD_{600} was > 0·5) in 20 mmol KH_2PO_4 (pH 7·0) buffer containing 1 mmol $MgCl_2$ and adjusted at 0·375 OD_{600} in the same buffer. In each microplate well, the bacterial suspension was fixed at 0·18 OD_{600} and nitrocefin (0·2 μg ml^{-1} final concentration) was added to start the enzymatic reaction. The microplate was immediately placed into an Infinite M200 pro Tecan microplate reader (Tecan), and OD were monitoring at 490 nm for 1 h (Matsumoto *et al.* 2011) to follow nitrocefin hydrolysis.

Preparation of membrane fractions, immunoblotting

Total protein fractions were prepared from bacterial exponential culture (1 OD_{600}), in the presence of respective concentration of preservatives for insusceptible strains. Cell pellets were solubilized at 96°C for porins analysis and 37°C for efflux pumps analysis. Bacterial membrane fractions were prepared from exponential cultures as previously described (Ghisalberti *et al.* 2005). SDS-PAGE and immunoblot methods were carried out as previously reported using polyclonal antibodies directed against OmpA, denatured porins, AcrA, AcrB and TolC efflux pump components (Simonet *et al.* 1996; Ghisalberti *et al.* 2005). Immunoblots were stained by colorimetric detection after incubation with alkaline-phosphatase-conjugated secondary antibodies (Ghisalberti *et al.* 2005).

Extraction of flagella and immunodetection

Bacteria were recovered for LB agar plates, containing respective concentrations of preservatives for insusceptible strains, and resuspended at 1 OD_{600} in phosphate buffer (0·1 mmol, pH 7·4). Cell suspension was pushed through syringe needles (0·8*40 nm) 20 times to shear the flagellar propellers (Lefèvre *et al.* 2010). Sheared cells were centrifuged at 14 000 ***g*** for 10 min at 4°C. Supernatant was precipitated with a final concentration of 15%

trichloracetic acid (TCA), pelleted and washed twice in acetone 90% by centrifugation (14 000 ***g***, 10 min, 4°C). In the next step, these pellets were resuspended in SDS-PAGE loading buffer and heated for 10 min at 96°C (Ghisalberti *et al.* 2005). Antibodies anti-H7 flagellin (Difco, Detroit, Mich) were used at 1 : 20 000 fold dilution for immunoblot analysis (Pradel *et al.* 2003). Immunoblots were stained by chemiluminescence detection after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies.

Mass spectrometry

The bands of interest were excised from the SDS-PAGE gel after staining and used for identification (Zhang *et al.* 2012). Tryptic digestion experiments of 1D bands were performed by a robotic sample preparation workstation (Freedom EVO 100, TECAN Männedorf, Switzerland) following multiple steps: washes, reduction and alkylation, digestion by trypsin (Sigma, St. Louis, MO, USA proteomics grade with 0·025% ProteasMax) and extraction of tryptic peptides.

Tryptic peptides were separated and analysed using a positive ion mode and two scan events with a LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Walthan, MA, USA) equipped with a nanospray ion source and coupled to a nano UPLC Ultimate NCS3500 (Dionex Sunnyvale, CA, USA). Spectra were processed by Proteome Discoverer software (Thermo Fisher Scientific), and the identification protein search was performed by MASCOT (*in situ* licence) with Database : NCBInr 2012 (17 333 135 sequences), Taxonomy : Bacteria (Eubacteria) (10 365 170 sequences). Proteins were considered as identified by 2 unique peptides with ion significance threshold $P < 0{\cdot}05$ (>44 individual MASCOT ion score) or 5 consecutive b and y ions.

Real-time efflux

The efflux mechanism in bacteria has been implicated in exhibiting a significant alteration of susceptibility for antibiotics and biocides (Nikaido and Pagès 2012; Ortega Morente *et al.* 2013). In Gram-negative bacteria, several efflux pumps have been identified in biocide-resistant strains (Ortega Morente *et al.* 2013). To determine and compare the activity of efflux pumps present in the various resistant derivatives and the parental strain, a method previously described by Lieutaud *et al.* (2013) was employed. This method makes it possible to follow the expel rate of a dye previously accumulated in the bacterial cell when an energy source (glucose) for the active efflux is added to the suspension (Brunel *et al.* 2013; Lieutaud *et al.* 2013). Briefly, for each strain, the different derivative-resistant strains inoculated with 20 ml of MHB were grown for 18 h at 37° C in the presence of respective preservative concentrations. All the subsequent steps were performed at room temperature. The cells were recovered by centrifugation and washed once in 20 mmol KH_2PO_4 buffer (pH 7·0) containing 1 mmol $MgCl_2$ (PPB). After a second centrifugation, the cell pellet was resuspended and adjusted to 0·25 OD_{600} in PPB. 1,20-Dinaphthylamine (1,2′ DNA) (TCI-Europe SA, Zwijndrecht, Belgium) and carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma) were added at 32 and 5 μmol, respectively. The incubation was then performed overnight at 37°C without shaking. Aliquots were taken from the cell suspension, and bacteria were recovered by centrifugation (4000 ***g***, 20 min) and resuspended in the same volume of PPB. The aliquot was then loaded (100 μl per well) into 96-well Greiner microplate (Greiner, Courtaboeuf, France), which was immediately placed into an Infinite M200 pro Tecan microplate reader. In the conditions used, the highest signal was obtained using an excitation wavelength $OD_{370\ nm}$ and an emission $OD_{420\ nm}$. All the recordings were performed at a gain setting of 65. The fluorescence of cell suspension was observed for 150 s, and the efflux of 1,2 DNA was then triggered by rapid energization of cells by adding glucose (50 mmol final concentration), monitoring the fluorescence for another 450 s.

Results

Susceptibility of preservatives adapted strains to disinfectants

Table 2 presents the different MIC and MBC results against the disinfectants that are generally used in the cosmetic industry. Peroxyacid, ADD/PHMC and laurylamine dipropylenediamine used in the disinfection step were assayed. Susceptibilities were determined on the parental strain *Ent. gergoviae* ATCC 33028, their respective MIT-CMIT- and triclosan-resistant derivatives. The industrial isolates, *for example* 23–26, have been previously tested (Périamé *et al.* 2014). MICs were tested in MH medium and MBC in Eugon LT medium. D1, D2 or D4 corresponds to 1, 2 or 4 days incubation, respectively. The use of D1, D2 and D4 allowed us to check the possibility of a delayed restart due to the presence of persister cells. Successive enumerations were performed according to ISO standards. No significant MIC differences were observed with MIT-CMIT- and triclosan-adapted strains compared to the parental strain (Table 2). For laurylamine dipropylenediamine present in Eugon LT, MBC was increased 8–16 times for MIT-CMIT- and triclosan-resistant derivatives compared to the parental strain. No significant MBC differences were measured for the 3 days.

Table 2 MICs and MBCs of disinfectants for the parental strain and MIT-CMIT (M1-M4) or triclosan (T1-T3)-resistant derivatives

	ADD/PHMC (%)		Peroxyacid (%)		Laurylamine dipropylamine (%)	
	MIC	MBC	MIC	MBC	MIC	MBC
Time	D1	D1, D2, D4	D1	D1, D2, D4	D1	D1, D2, D4
ATCC 33028	0·015	0·03	0·007	0·015	0·06	0·06
M1	0·015	0·06	0·007	0·015	0·06	0·125
M2	0·015	0·06	0·015	0·015	0·06	0·5
M3	0·015	0·06	0·015	0·015	0·06	1
M4	0·015	0·03	0·015	0·015	0·06	1
T1	0·015	0·03	0·015	0·015	0·06	0·5
T2	0·015	0·015	0·007	0·015	0·06	0·5
T3	0·015	0·03	0·007	0·015	0·06	0·5

Susceptibility to antibiotics, preservatives and chemicals (EDTA, DOC)

The MICs for polymyxin B, nalidixic acid and β-lactams (imipenem, cefepime, ceftazidime) did not differ substantially between the resistant derivatives and their parental strain (Table 3). In the same way, no MIC variations were observed for triclosan and MIT-CMIT between ATCC, derivatives and cosmetic strains (no more than one dilution difference which is considered as no significant). Addition of PABN caused an increase in antibiotics tested susceptibility by no more than one dilution factor as for nalidixic acid of ATCC 33028 (parental strain) and resistant strains (M1-M4, T1-T3), indicating with the criteria of the 3 significative dilution factors that no PAβN-susceptible efflux could be detected. Only the polymyxin B susceptibility was increased in the presence of PAßN for all strains suggesting a similar synergistic effect on the *Ent. gergoviae* susceptibility (eightfold, respectively). This PAßN concentration (20 mg l^{-1}) has been previously used to restore antibiotic susceptibility in *Ent. aerogenes*- and *Klebsiella pneumoniae*-resistant strains (Mamelli *et al.* 2009; Pagès *et al.* 2009; Kuete *et al.* 2011). In same assays, no effect was obtained for ß-lactam in the presence of PAßN and a subinhibitory concentration of cloxacillin (data not shown). Interestingly, the susceptibility to EDTA reached 80 mmol for MIT-CMIT- and triclosan-resistant derivatives strains that corresponded to about 16-fold increase compared to the parental strain. This susceptibility decrease could be associated with an adaptive resistance to EDTA-chelating action and outer membrane destabilizing strategy. The destabilization of the LPS layer induced by removal of divalent cations by EDTA could be reduced in adaptive strains, resulting in a preserved integrity of the outer membrane. No modifications in the susceptibility were observed with polymyxin B, a permeabilizing agent in the absence or in the presence of EDTA. In addition, a decrease of DOC susceptibility was also observed in the resistant strains. Interestingly, various papers reported a relationship between LPS modification and the susceptibility of Gram-negative bacteria to EDTA and detergents (Bell *et al.* 1991; Liu *et al.* 2012). Together, these data suggest the involvement of outer membrane and particularly LPS modifications in bacteria biocides adaptation as precedently observed (Chapman *et al.* 1998).

Impact of preservatives and disinfectants on the permeability of the outer membrane

Many biocide molecules exhibit a membranotropic effect inducing an alteration of membrane permeability (Ortega Morente *et al.* 2013). Hence, we were inclined in knowing if a modification of the outer membrane barrier is liable for the emergence of resistant strains after biocide exposure. The assays using the determination of ß-lactamase release have been described as powerful tools to follow the possible change of membrane physiology (Fadli *et al.* 2012). In this assay, nitrocefin is hydrolysed by the periplasmic ß-lactamase of Gram-negative bacteria, and the detection of chromogenic product is an indicator of the outer membrane (OM) permeability (Jones *et al.* 1982).

We first tested the OM permeabilizing potential of five preservatives (methylisothiazolinone, MIT-CMIT, pentylene glycol, levulinic acid, triclosan) and one disinfectant (peroxyacid) on the parental ATCC strain. Only the peroxyacid showed an increase of nitrocefin hydrolysis due to a permeabilizing effect obtained with concentrations between 0·20% and 0·5%, which corresponds to the concentration range of use in cosmetic industries (Fig. 1).

Regarding the resistant strains, the amount of hydrolysed nitrocefin was not significantly changed in MIT-CMIT (M1-M4)- and in triclosan (T1-T3)-resistant isolates

Table 3 Susceptibilities of the *Enterobacter gergoviae* ATCC 33028 strain and its derivative-resistant strains to EDTA, DOC (deoxycholate), MIT-CMIT, triclosan and different antibiotics (polymyxin, nalidixic acid, imipenem, ceftazidime, cefepime) with or without efflux pump inhibitor (PAβN)

strains	DOC (μg ml^{-1})	EDTA (mmol)	POL (μg ml^{-1})	POL+ EDTA	POL+ PAβN	Nal (μg ml^{-1})	Nal+ PAβN	IMI (μg ml^{-1})	IMI+ PAβN	CAZ (μg ml^{-1})	CAZ+ PAβN	CEF (μg ml^{-1})	CEF+ PAβN	MIT-CMIT (%)	MIT-CMIT + PAβN	Triclosan (μg ml^{-1})	Triclosan + PAβN
ATCC 33028	8	5	1	1	0·12	32	32	0·25	0·5	1	2	0·5	0·5	$1{\cdot}5^{E-4}$	$1{\cdot}5^{E-4}$	2·5	2·5
M1	>16*	80	1	1	0·25	32	32	0·25	0·5	2	2	1	0·5	$1{\cdot}2^{E-3}$	$1{\cdot}2^{E-3}$	2·5	2·5
M2	>16*	80	1	1	0·25	64	32	0·25	0·5	2	2	0·25	0·5	6^{E-4}	6^{E-4}	2·5	2·5
M3	>16*	80	2	1	0·12	64	32	0·125	0·5	2	1	1	0·5	6^{E-4}	6^{E-4}	2·5	2·5
M4	>16*	80	2	1	0·12	64	32	0·25	0·25	2	1	1	0·5	6^{E-4}	6^{E-4}	2·5	1·25
T1	>16*	80	1	0·5	0·12	32	32	0·25	0·25	2	2	0·25	1	$1{\cdot}5^{E-4}$	$1{\cdot}5^{E-4}$	5	2·5
T2	>16*	80	1	1	0·12	64	32	0·25	0·25	2	1	0·25	0·5	$1{\cdot}5^{E-4}$	$7{\cdot}5^{E-5}$	10	10
T3	>16*	80	1	1	0·12	64	32	0·25	0·5	2	1	1	0·5	$7{\cdot}5^{E-5}$	$7{\cdot}5^{E-5}$	20	20

In combination with polymyxin B, EDTA was tested at 1 mmol.
*For DOC, complete solubilization was not possible beyond the concentration >16 μg ml^{-1}.

(data not shown). This suggests that no important alteration of the outer membrane permeability promoting the release of hydrolysed nitrocefin could be present in the resistant isolates.

Outer and inner membrane proteins in the various strains

Analysis of total bacterial proteins by SDS-PAGE showed no difference in the migration of molecules in the size range of 30–45 kDa molecular weights. Similarly, the immunodetections of OmpA, porins, and components of efflux pump AcrA, AcrB and TolC reported no variation in the signal intensity or in the migration of these key membrane proteins (Fig. 2). Interestingly, after SDS-PAGE evaluation of the bacterial compartments, various differences were observed in the profile: in the OM fraction of the resistant strain, (i) we observed an additional band migrating around 35-kDa mark, (ii) a more intense upper band at about 40 kDa and (iii) the appearance of a slightly intermediate band between them. The three instances have been pointed as c, d, e in Fig. 3. Various differences were also observed on the profiles of *Ent. gergoviae* strains, eg23 and eg26, isolated from contaminated cosmetics, for which we have previously reported an insusceptibility for various preservatives (Périamé *et al.* 2014). They exhibited three bands instead of the two detected in the parental strain ATCC 33028. The 35- to 45-kDa part of the SDS-PAGE exhibited some similarity with the profile of the MIT-CMIT-resistant strains.

Flagellin and peroxiredoxin identification in various strains

Mass spectrometry analyses were carried out to identify the proteins detected in the SDS-PAGE patterns of resistant strains. The respective six bands, a to f (Fig. 3), were excised from the gels, and the proteins were retrieved and used for mass spectrometric determinations.

The analysis identified several proteins—the lower band as OmpA—the upper band one for the parental strain, and two bands for the derivative resistant strains were identified as flagellin protein (Table 4). A higher molecular weight flagellin was detected in the resistant strain M1. Immunodetection using anti-H7 flagellin matched with two bands: one located in the OM fraction, close to 40 kDa for M1, and one identified in the supernatant fraction, close to 38 kDa. The immunoreactive protein was observed in the M1-resistant strain, the triclosan-resistant derivatives (T1-T3) and strains isolated from cosmetic products (eg23 and eg26) (Fig. 4). Furthermore, a 25-kDa product was observed in the cytosolic fraction of the M1 strain. Six peptides showing 46%

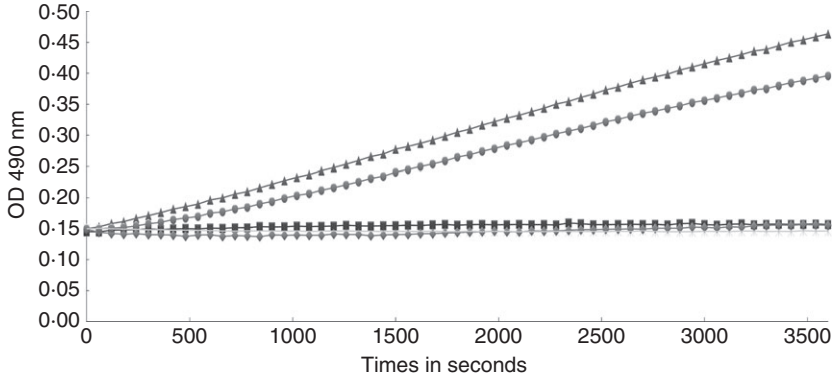

Figure 1 ß-lactamase activity measured in the ATCC 33028 treated by various compounds. Cells were resuspended in fresh medium in the presence of methylisothiazolinone, MIT-CMIT, pentylene glycol, levulinic acid, triclosan and peroxyacid. The bacterial suspensions were incubated in microplate well, and nitrocefin was added to start the enzymatic measure. The β-lactamase activity was determined by following nitrocefin hydrolysis on chromogenic compounds at λ 490 nm for 1 h (Matsumoto *et al.* 2011). (▲) peroxyacid 0·4%; (●) peroxyacid 0·2%; (■) methylisothiazolinone 0·01%; (♦) MIT-CMIT 0·012%: (×) triclosan 20 mg l^{-1}.

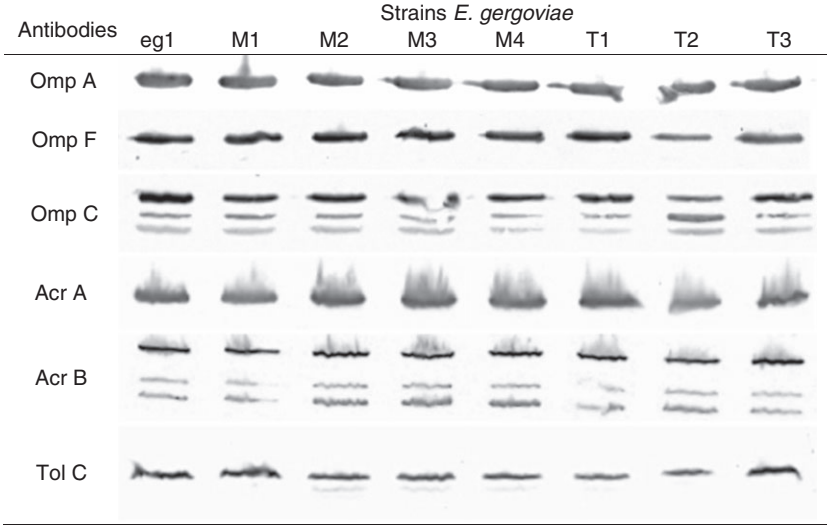

Figure 2 Immunodetection of major outer membrane proteins and components of efflux pump AcrAB-TolC, Total bacterial proteins were separated by SDS-PAGE, electrotransferred onto nitrocellulose and analysed by immunoblotting as previously described (Simonet *et al.* 1996; Ghisalberti *et al.* 2005). Antibodies directed against OmpA, OmpC, OmpF, AcrA AcrB, and TolC were used. Immunoblots were stained by chemiluminescence detection after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Only the relevant part of the sheet was presented.

sequence coverage (MASCOT search on NCBInr Database reduced to Bacteria (15 365 170 entries) were obtained (Table 4). The mass spectrometry analyses fitted in with an antioxidant enzyme, peroxiredoxin that controls cytokine-induced peroxide levels, for this 25-kDa protein.

Real-time efflux

Bacteria are able to expel a large variety of compounds such as antibiotics, biocides, detergents or dyes (Nikaido and Pagès 2012). 1,2 DNA, a fluorescent dye, is a substrate of the AcrAB efflux pump belonging to RND family, and it has been used to monitor the bacterial efflux. A decrease in the dye's fluorescence intensity allows the assessment of the activity of active drug pump (Brunel *et al.* 2013; Lieutaud *et al.* 2013). When the assays were carried out, the resistant strains showed a significant variation in the efflux activity compared to those obtained with the parental strain (Fig. 5). An efflux activity corresponding to about 75–60% of the level determined in the

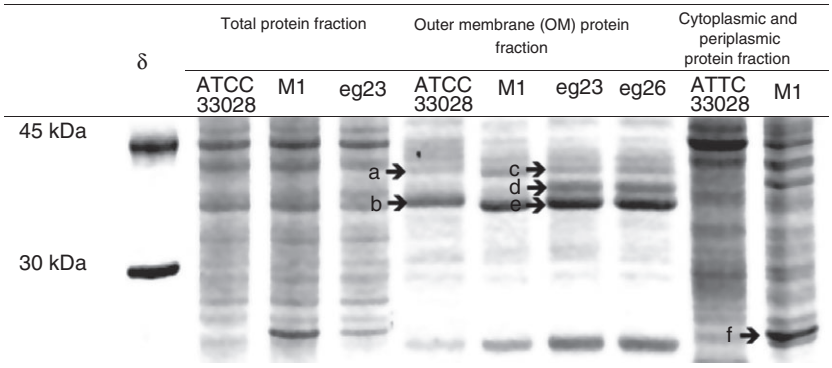

Figure 3 SDS-PAGE profiles for total membrane proteins, outer membrane proteins and soluble proteins. Bacterial membrane fractions were prepared from exponential cultures as previously described (Ghisalberti *et al.* 2005). SDS-PAGE were carried out and stained as previously reported (Ghisalberti *et al.* 2005). The total membrane proteins, outer membrane proteins and cytosolic proteins (cytoplasmic + periplasmic proteins) were indicated. δ corresponds to the standard molecular weight; a, c, d = flagellin; b, e = outer membrane protein A (OmpA); f = peroxiredoxin.

Table 4 Identification of detected proteins in the resistant strains presented in SDS-PAGE outer membrane proteins

Sample	Gi	Mascot Score	Mass (Da)	Matches	Sequences	Name	Coverage (%)
a	gi|56383043	555	36 326	15	7	Flagellin [*Escherichia coli*]	27
b	gi|376387855	666	38 239	107	11	Outer membrane protein A *[Klebsiella oxytoca 10-5243]*	22
c	gi|56383043	2156	36 326	72	7	Flagellin [*E. coli*]	27
d	gi|56383043	1147	36 326	36	4	Flagellin [*E. coli*]	20
e	gi|317491503	277	37 996	102	7	OmpA transmembrane domain-containing protein [*Enterobacteriaceae bacterium 9-2-54FAA*]	24
f	gi|345298292	536	20 792	21	19	Peroxiredoxin [*Enterobacter asburiae LF7a*]	46

Sample: 'a, b, c, d, e, f' correspond to the names of protein band identified on the SDS-PAGE presented in the Fig 3.
Matches, number of peptides; Sequences, number of different peptides.

parental strain was observed for MIT-CMIT-resistant strains (M1-M4) that produced very similar curves. Interestingly, a stronger reduction was observed with the triclosan-resistant strains (T1-T3) with only 35–18% of the efflux measured compared to the parental strain (Fig. 5). These results suggest that a reduction of the dye efflux efficacy was reached in the resistant strains.

Discussion

The objective of this work was to understand the *Ent. gergoviae* adaptive mechanisms to preservatives used in the case of recurrent contaminations. To this aim, we obtained and characterized resistant isolates which are able to grow in the presence of increased concentrations of preservatives. Different profiles were observed depending on the preservatives used during the selection as illustrated by the respective MIT-CMIT and triclosan MICs and the laurylamine MBC measurements.

Importantly, the resistant strains selected with increased concentrations of MIT-CMIT or triclosan presented no variation in antibiotic susceptibility in Mueller–Hinton broth containing twofold serial dilutions of each antibiotic. This indicated that no cross-adaptive mechanisms between these two preservatives and antibiotics were selected or expressed such as the activation of a common efflux system or membrane impermeability (AcrAB overproduction, loss of porins). Various studies concerning biocide adaptation, with a special attention to triclosan, have produced contradictory results about the relationship between biocides and antibiotics and the involvement of general mechanisms such as the efflux (Chuanchuen *et al.* 2001; Condell *et al.* 2012; Sheridan *et al.* 2012; Rensch *et al.* 2013). Moreover, regarding nalidixic acid, MIT-CMIT and triclosan, PAßN used at a concentration previously reported to block efflux activity in *Ent. aerogenes*-, *Kl. pneumoniae*- and *Escherichia coli*-resistant strains (Mamelli *et al.* 2009; Pagès *et al.* 2009;

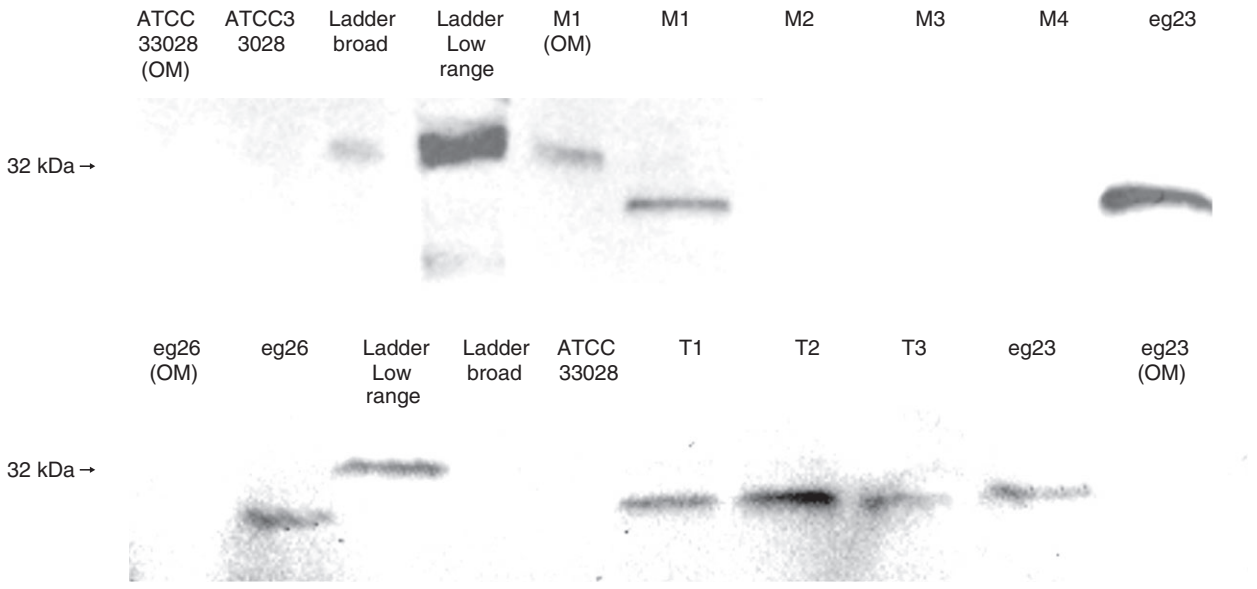

Figure 4 Immunodetection of flagellin. ATCC strain, eg23 isolate and resistant derivates MIT-CMIT (M1 to M4) were cultivated. Bacterial fractions were prepared from exponential cultures and SDS-PAGE were carried out and stained as described in Materials and Methods. The immunodetection was carried out with polyclonal antibody against H7 flagellin of *Escherichia coli*. Immunoblots were stained by chemiluminescence detection after incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies. Only the relevant part of the sheet was presented.

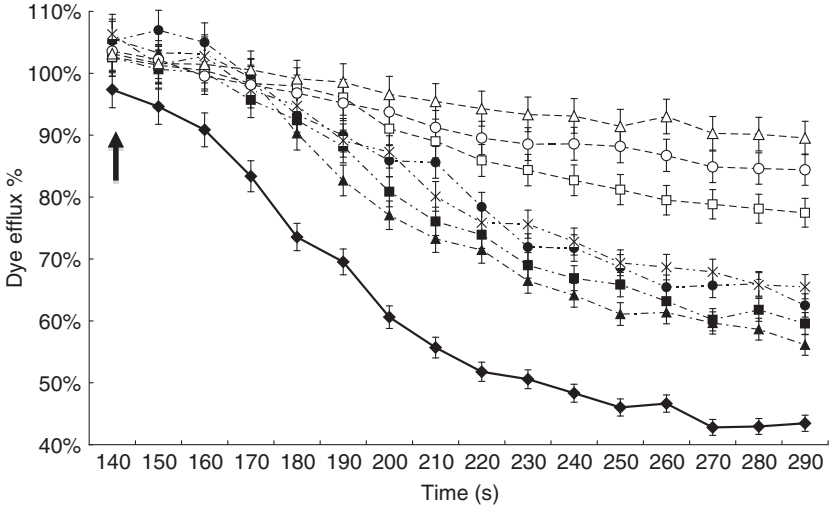

Figure 5 Real-time efflux kinetics for the parental strain ATCC33028 and all derivates variants MIT-CMIT (M1-M4) and triclosan (T1-T3). The fluorochrome 1, 2 DNA was used as a substrate of the efflux system as previously reported (Brunel *et al.* 2013; Lieutaud *et al.* 2013). The time is in seconds and fluorescence in % was normalized on eg1 kinetic. At 150 s, energization of cells was induced by adding 5 μl of 1 M glucose (indicated by an arrow in the figure), and the fluorescence decrease was monitored to measure efflux activity. Standard deviations were displayed. Strains and derivates: (♦) ATCC 33028, (■) M1, (●) M2, (▲) M3, (×) M4, (□) T1, (○) T2, (△) T3.

Kuete *et al.* 2011) did not increase the respective drug activity whatever the strain tested. In addition, no significant change was noted for ß-lactam molecules, which have been reported to be substrates for *Kl. pneumonia* AcrAB efflux pump (Pagès *et al.* 2009). Moreover, immunodetections of the major pump AcrAB-TolC reported no significant variation in the expression level of efflux components and analysis of ß-lactamase activity indicated no change in the outer membrane permeability of resistant mutants. Consequently, in the resistant derivatives, the preservative insusceptibility may use other mechanisms than those involved in antibiotic resistance such as

AcrAB-TolC efflux pump as previously reported (Condell *et al.* 2012; Rensch *et al.* 2013; Rushton *et al.* 2013).

Interestingly, no alteration in porins expression and in ß-lactams susceptibility was observed, indicating that a change in the porin-associated permeability is not involved in the various resistant strains, in contrast to previous reports (Chapman *et al.* 1998; Ortega Morente *et al.* 2013). Concerning outer membrane proteins and bacterial adaptation to isothiazolinones, different results have been reported in *Pseudomonas aeruginosa*. It was described that the *Ps. aeruginosa* adaptation to MIT and CMIT was associated with the decrease of a 35-kDa outer membrane protein named T-OMP and with a decreased susceptibility towards the individual components of MIT-CMIT (Brözel and Cloete 1994). Moreover, T-OMP production was observed *de novo* after removal of the isothiazolone biocides, while the treated cultures still exhibited some residual tolerance towards these compounds. The authors suggested that T-OMP expression was linked to isothiazolone; however, T-OMP itself was not the cause of the decreased susceptibility (Winder *et al.* 2000). It was concluded that the T-OMP expression may be specific for thiol-interactive biocides in *Ps. aeruginosa*. No additional studies have been carried out to determine the biological function of T-OMP and its possible likeness with OMPs in Enterobacteriaceae.

These results suggest that the exposure of *Ent. gergoviae* to MIT-CMIT or triclosan under the conditions used in this study did not activate a global control cascade as previously reported in the case of *Ent. aerogenes* and imipenem exposure that triggers a Mar operon response (Davin-Regli *et al.* 2008). This is interesting to mention that a stress response involving stress regulons has been previously reported for *E. coli* and *Ps. aeruginosa* during preservative exposure (Abdel-Malek *et al.* 2002; Yu *et al.* 2010; Zhou *et al.* 2014).

In contrast, MIT-CMIT- and triclosan-resistant derivatives were less susceptible to laurylamine dipropylenediamine compared to the parental strain. This indicates that the mechanisms selected in the derivative strains can provide a cross-insusceptibility towards some preservatives and disinfectants. This bacterial adaptation may impair the activity of various disinfectants used in practice to address *Ent. gergoviae* contaminations. The MIT-CMIT is an isothiazolinone biocide used by the cosmetic company for the disinfection of cosmetic production and distribution systems, destructing protein-thiols and producing free radicals (Williams 2006). MIT and CMIT interact *via* redox reaction with thiols to form disulphites, cystine and biocide dimers (thio-acyl chloride) highly reactive in the case of CMIT (Collier *et al.* 1990a, b, 1991). The thio-acyl chloride is associated with the antimicrobial activity of isothiazolinones by reacting with glutathione or amines (Collier *et al.* 1990b). The ADD/PHMC combination and the laurylamine dipropylenediamine have surfactant properties. Biguanides seem to cause a strong destabilization of the membrane causing leakage of cytoplasmic contents (Broxton *et al.* 1984; Ikeda *et al.* 1984; Allen *et al.* 2006). Peroxygen molecules have strong oxidative property that attack essential cell components and cell membranes and additionally produced active hydroxyl radicals. However, peroxyacid effects seemed less effective on the MIT-CMIT- or triclosan-resistant strains compared to the parental strain, suggesting that membrane changes provide some protection towards peroxygens molecules in resistant derivatives.

Analyses of membrane proteins potentially involved in the permeability adaptation such as efflux pumps or porins showed no significant differences between derivative resistant strains and the parental strain. The main difference observed in the M1 adapted strain and strains isolated from cosmetics corresponds to a significant expression of a flagellin protein identified by mass spectrometry. Overexpression of flagellin components has been previously reported in *E. coli* triclosan-resistant mutants (Bailey *et al.* 2009; Sheridan *et al.* 2013). Interestingly, in these strains that exhibit a modification of flagellin expression, we also observed a decrease in EDTA and DOC susceptibility, indicating involvement of outer membrane as a dynamic and permeability barrier face to preservatives as mentioned for *Ps. aeruginosa* (Zhou *et al.* 2014). In a recent publication, Kirkpatrick and Viollier reported the intricate relationship between efflux system and flagella functional assembly (Kirkpatrick and Viollier 2014). This original mechanism involved in drug insusceptibility is associated with the control of flagellin expression in *Caulobacter crescentus*. This study presents some similarities with the results associated with *Ent. gergoviae* triclosan-resistant mutants, for example expression of flagellin, decrease of dye efflux and envelope modification. We hypothesized that this variation could be associated with modifications of bacterial surface or in the modification of the energy level of bacterial membrane (Thiolas *et al.* 2005; Kastbjerg *et al.* 2014). Interestingly, the triclosan-resistant strains exhibit a noticeable difference in the dye efflux that requires a membrane energy source as previously demonstrated (Lieutaud *et al.* 2013). In the MIT-CMIT-resistant strain M1, an overproduction of peroxiredoxin has been observed. Peroxiredoxins are cysteine-based peroxidases that act as redox sensors and biomarkers of oxidative stress. They are detoxifying enzymes expressed in a wide range of eubacteria for reactive species as hydroperoxides (Wood *et al.* 2003; Hall *et al.* 2009). The cysteines are oxidized to reduce peroxides by forming an intermolecular disulphide bond, and the disulphide-bonded peroxiredoxins are subsequently

reduced and reactivated by thiol-containing reductants such as alkyl hydroperoxidase subunit F (AhpF) and thioredoxin (TrxA). Interestingly, the isothiazolones are biocides which have a growth inhibition activity associated with a thiol-interactivity (Collier *et al.* 1990a,b, 1991). Moreover, thiol-containing compounds such as cysteine, which is a thiol amino acid, are able to neutralize activity of CMIT (Collier *et al.* 1990a). Consequently, there is an evident relationship between isothiazolinones activity and overexpression of peroxiredoxin. AhpC (alkyl hydroperoxidase subunit C), one of the best characterized 2-cysteine peroxiredoxins, utilizes AhpF as a reductant to compose an NAD(P)H-dependent peroxidase system. Expression of AhpC is activated by OxyR, which is a central regulator of the oxidative stress response in a number of bacteria (Hall *et al.* 2009; Zhou *et al.* 2014). Interestingly, it has been recently demonstrated that *Burkholderia cepacia* strains, resistant to isothiazolinones, overexpressed the transcriptional activator OxyR (Zhou *et al.* 2014).

The present results established an additional relationship between the isothiazolinones biocides, the cellular interactions response including bacterial oxidative stress answer and detoxifying enzymes regulation and the bacterial tolerance to biocides.

To conclude, it is clear that the resistant strains obtained in this study exhibited no cross-adaptive mechanism altering the antibiotic activity; in contrast, a cross-insusceptibility towards preservatives and disinfectants was observed. It is important to mention that no increase of efflux activity or modification of porins level was detected in the various MIT-CMIT- and triclosan-resistant strains. Interestingly, several insusceptible strains and the two strains issued from contaminated cosmetics exhibited a strong modification of bacterial envelope with an overproduction of flagellin, but the porins and OmpA expression was uniform. This suggests that the same adaptive mechanisms may be present in the laboratory-induced insusceptible strains and the industrial isolates.

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Conflict of Interest

No conflict of interest declared.

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