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Enterobacter gergoviae and the prevalence of efflux in parabens resistance

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***Objectives*: In order to characterize the mechanism involved in parabens resistance, we studied 13 *Enterobacter gergoviae* collected from diverse cosmetic formulations containing parabens as preservatives and 10 isolates from clinical or industrial sources.**

***Methods*: RAPD and ERIC-PCR were employed and compared for the epidemiological typing. To study antibiotic and paraben susceptibility, the standard disc diffusion method and the 2-fold dilution method in Luria–Bertani medium were used. Characterization of porins was performed using immunodetection with polyclonal antibodies. Resistance mechanisms against parabens membrane permeabilization were evaluated by measuring K^+ efflux using a specific electrode. *mar* regulon identification and comparison were carried out.**

***Results*: Epidemiological typing confirmed that most of the cosmetic formulations were contaminated by unrelated strains. All of the *E. gergoviae* strains presented high methylparaben MICs, ranging from 1 to 3.8 g/L, values that were 2–5 times higher than for *Escherichia coli* or *Enterobacter aerogenes*, even in strains overexpressing MarA. These MICs decreased in the presence of phenylalanine arginine β-naphthylamide, pinpointing efflux as a major mechanism of parabens resistance even in *E. gergoviae* clinical strains.**

***Conclusions*: This is the first report showing the role of active efflux in the parabens resistance in *E. gergoviae*, a mechanism that may explain its frequent isolation in parabens-containing cosmetics compared with other enterobacterial species. Paraben efflux seems to be regulated by a *mar*-independent process in *E. gergoviae*.**

Keywords: preservatives, resistance, cosmetics, *mar*, potassium efflux, RAPD

Introduction

Parabens are used as antimicrobial preservatives, particularly in cosmetic products; furthermore, they are likely to have multiple targets and biological effects.[1] Little is known concerning the bacterial resistance to parabens. An esterase named PrbA, which hydrolyses high concentrations of parabens into 4-hydroxybenzoic acid, has recently been identified.[2]

Enterobacter gergoviae is rarely isolated in clinical laboratories and it is generally susceptible to antibiotics however, some cosmetic laboratories were concerned about the contamination of their different cosmetic formulations, containing a combination of parabens as preservatives, by *E. gergoviae*.

From this study it may be concluded that most of the cosmetics were contaminated by unrelated strains showing a natural paraben efflux mechanism that does not affect antibiotics. The *marRAB* operon of *E. gergoviae* is described, but the efflux of parabens is independent of *mar*-mediated regulation. Considering that *E. gergoviae* is naturally resistant to parabens at the concentrations used in cosmetics, the contamination of cosmetics containing parabens by *E. gergoviae* is a matter of concern.

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Materials and methods

Strains and plasmid

Over the period 1992–2000, 13 *E. gergoviae* were isolated from diverse cosmetic formulations from four microbiological laboratories of cosmetic control in France. One or more parabens in combination were present in concentrations between 0.15 and 1.00% (w/w). We selected 10 other *E. gergoviae*: one for the challenge test, two from industrial products and seven clinical strains (ATCC 76.1T, 76.2, 79.51, 33426 and 33428 and CIP 104955 and 104981). Isolates were confirmed to be *E. gergoviae* by the API 20 E system and Biotype-100 carbon source strips (bioMérieux, Marcy-l'Étoile, France). *Ea* 27 strain is a multidrug-resistant (MDR) clinical *Enterobacter aerogenes*, used as a control for its efflux resistance mechanism, that overexpresses the AcrAB-TolC efflux system.[3] Plasmid p9, a multicopy plasmid containing the cloned *marA–Escherichia coli* gene (Ap^r; 2.2 kb *marAB–Eco* insert in pBR322; *marAB* expressed from the tetracycline promoter), was introduced and expressed previously in *E. aerogenes* ATCC 13048 and *E. coli* DH5α and in the present study in *E. gergoviae* CIP76-1.[4] All bacterial strains were grown in Luria–Bertani (LB) agar or in Mueller–Hinton (MH) medium for 24 h at 30°C.

Epidemiological typing

The isolates were investigated by RAPD with primer AP12H and ERIC-PCR with primer ERIC2 as described previously.[5]

Antibiotic and paraben susceptibility testing

Antibiotic susceptibility was determined by the standard disc diffusion method on MH agar (bioMérieux). The MICs of methylparaben were determined on a methylparaben gradient:[6] each strain was spread onto successive gradient LB agar plates, containing concentrations ranging from 0.01 to 4 g/L. MICs of methylparaben were confirmed by a standard 2-fold dilution method in LB medium.[3] Among the 23 strains, 6 were selected for their origin, expression of the esterase (EG3 and EG7)[2] and antibiotic resistance: strains EG2, EG3, EG4 and EG7 were isolated from cosmetics and EG17 and EG18 were of clinical origin. Growth in the presence of 1 g/L propylparaben or 0.8 mg/L salicylate and their influence on antibiotic resistance levels were evaluated on LB agar plates. Three successive subcultures preceded antibiotic susceptibility evaluation by the disc diffusion method. Each result was an average of two experiments.

SDS–PAGE and immunodetection of porins

Exponential bacterial cells grown in LB broth were collected. Bacterial cell pellets were solubilized in loading buffer at 96°C, and samples (amounts corresponding to 0.02 optical density units at 600 nm) were loaded onto SDS/polyacrylamide gels (0.1% SDS/10% polyacrylamide) as described previously.[3,7] Electrotransfer to nitrocellulose membranes was performed in the presence of 0.05% SDS to achieve complete transfer of porins. An initial saturating step with Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 8) containing 10% skimmed milk was carried out overnight at 4°C. The nitrocellulose membranes were then incubated in the same buffer supplemented with 0.2% Triton X-100 for 2 h at room temperature with polyclonal antibodies directed against denatured porin monomers and the antipeptide F4 directed against the internal porin L3 loop. Polyclonal antibodies directed against the *E. coli* porins were able to recognize all enterobacterial porins.[3] After successive washings in the same buffer, the detection was performed with alkaline phosphatase-conjugated AffinitiPure goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch, West Grove, PA, USA).

Potassium efflux measurements

It has been shown that propylparaben is able to open bacterial mechanosensitive channels, allowing the leakage of cytoplasmic content.[8] The impact of parabens on membrane integrity was assayed for strains EG4 and EG7. The potassium efflux measurements were carried out after addition of propylparaben (0.5 g/L final concentration).[9] Each result was the average of a minimum of two experiments.

DNA preparation, primers and PCR amplification

Preparation of DNA was performed using the hexadecyltrimethylammonium bromide method.[7] Primers M3 and M7 were designed from the sequence of the putative *mar* operon of *Klebsiella pneumoniae*, MCD and MR1 correspond to the most conserved sequences among different *mar* loci and EG2D and EG3R are specific for the *E. gergoviae mar* (*mar-Eg*) locus:[4] MCD, 5′-GGCCAAGGAACAGCGCCAC-3′; MR1, 5′-GCACCAGCACGCCGCGTT-3′; M3, 5′-GATCGCCTGCTCAATGACTAC-3′; EG2D, 5′-GACGCTATCACCATTCATAG-3′; EG3R, 5′-TTCTTAAACGTGCGCGTCA-3′; M7, 5′-TATGATTGAAATCAAACGGCG-3′. PCR amplifications were performed in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Boston, MA, USA) as described previously.[4]

DNA sequencing and data analysis

The sequences of the PCR products were determined with an ABI Prism 377 DNA sequencer with dye fluorescent terminators and the primers used in the initial PCR amplification as described previously.[4] The *marA-Eg* locus was submitted to the EMBL database and was given accession no. AY533026. Sequence comparison analysis was performed on the infobiogene server http://www.ncbi.nlm.nih.gov/BLAST.

Results and discussion

Genotypic characterization of E. gergoviae isolates

The RAPD and ERIC-PCR successfully typed all isolates and results indicated a good correlation between the two primers. All the 23 strains except G1–G2, G7–G8 and G9–G12 from cosmetics presented different RAPD types (data not shown). The identical strains within each pair were isolated from the same laboratory, but the year of isolation was different in two of the three cases.

Immunodetection of outer membrane proteins

Polyclonal antibodies allowed us to investigate the presence of porins in the various *E. gergoviae* strains. Antigenic related porins were observed in the 23 strains, irrespective of the level of paraben resistance (data not shown).

Antibiotic and paraben susceptibility

The 23 strains were susceptible to the antibiotics tested, except the clinical strains *E. gergoviae* 76.1 T and 76.2 (data not shown).

The effect on antibiotic resistance of subculturing in the presence of propylparaben or salicylate, a well-known *mar* inducer, was investigated. After subculturing in the presence of salicylate,

Table 1. Comparison of the inhibition zone sizes in the six *E. gergoviae* strains (EG2, EG3, EG4, EG7, EG17 and EG18) after subculturing in the presence of propylparben or salicylate and after transformation by plasmid p9 bearing *marA-Eco*

	Inhibition zone sizes (mm)							
Strain	AMP	TET	AMC	CEF	CHL	NOR	NAL	FEP
EG17	17	23	23	10	14	27	13	33
EG17 PPB	17	23	23	9	18	27	13	33
EG17 Sal	15	21	22	7	13	25	12	30
EG17(p9)	7	20	10	7	9	25	7	27
EG18	26	28	29	27	28	34	26	38
EG18 PPB	21	26	26	18	26	32	22	36
EG18 Sal	20	25	25	15	22	30	17	35
EG18 (p9)	7	21	10	7	9	25	7	27
EG2	24	27	27	22	23	35	25	38
EG2 PPB	19	26	24	11	21	34	25	37
EG2 Sal	18	27	24	9	13	34	19	36
EG2 (p9)	7	26	13	13	24	36	25	33
EG3	29	28	30	26	29	39	27	42
EG3 PPB	19	26	25	12	21	31	19	34
EG3 Sal	20	26	27	11	16	34	19	37
EG3 (p9)	7	22	10	7	11	23	9	25
EG4	27	28	30	25	27	37	28	41
EG4 PPB	24	26	27	15	26	36	22	37
EG4 Sal	20	25	27	13	18	32	17	37
EG4 (p9)	7	19	14	7	12	28	14	30
EG7	27	28	28	27	26	37	23	38
EG7 PPB	26	26	28	26	26	35	25	36
EG7 Sal	20	25	25	13	17	30	15	36
EG7(p9)	7	19	9	7	8	28	13	28

PPB, propylparaben; Sal, salicylate.
Antibiotics tested were as follows: AMP, ampicillin; TET, tetracycline; AMC, amoxicillin/clavulanic acid; CEF, cefalotin; CHL, chloramphenicol; NOR, norfloxacin; NAL, nalidixic acid; FEP, cefepime. Paper discs were used (6 mm diameter). According to the growth inhibition zones, which include the diameters of the discs, bacterial strains were classified as susceptible, intermediate or resistant following the guidelines of the French Antibiogram Committee (CA-SFM; available at http://www.sfm.asso.fr/).

Table 2. Capacity of phenylalanine arginine β-naphthylamide (PAβN) to restore susceptibility to methylparaben for determination of the involvement of an efflux mechanism in parabens resistance

		Methylparaben MIC (g/L)		
Strain	Esterase activity[a]	–PAβN	+PAβN (26.3 mg/L)	+p9
EG2	8.9	1.25	0.25	1.5
EG3	100	3.8	1	3.8
EG4	8.5	1.25	0.25	1.5
EG7	97.9	3.8	0.25	3.8
EG17	14	1	0.125	1.5
EG18	12.6	1.25	0.125	1.5
E. aerogenes 13048	0.5	ND	ND	
Ea 27	0.5	ND	ND	
E. coli DH5α	0.5	ND	0.5	

ND, not determined.
The six *E. gergoviae* selected strains were compared with *E. aerogenes* ATCC 13048, *Ea* 27 and *E. coli* DH5α. Influence of MarA-*Eco* on methylparaben susceptibility was investigated in strains transformed by p9 expressing MarA-*Eco*. Various concentrations of methylparaben, alone or in the presence of the efflux pump inhibitor PAβN at 26.3 mg/L, were tested using a 2-fold standard dilution method in LB medium.[3] Methylparaben MICs were obtained using a 2-fold standard dilution method in LB medium.
[a]Esterase activity: expressed as the percentage of 800 ppm of methylparaben (5.7 mM) degraded after 2 h of incubation by an exponentially growing culture.[2]

which is an *o*-hydroxybenzoic acid structurally analogous to parabens, all strains except EG2 presented a diminution of drug susceptibilities (Table 1). These results indicated a probable activation of the MDR phenotype mediated via MarA. Conversely, propylparaben, which is structurally analogous to salicylate, is not able to activate the MDR cascade mediated by *marA*, as demonstrated by the absence, or very low modifications, of antibiotic susceptibility. These results suggest little effect of parabens on the induction of MDR.

Transformation by the plasmid p9 induced a pleotropic diminution of antibiotic susceptibility, confirming the existence of a MarA-mediated response in *E. gergoviae* (Table 1). However, little effect was observed on methylparaben susceptibility (Table 2). MICs of methylparaben for *Ea* 27, *E. aerogenes* 13048 and *E. coli* DH5α transformed by p9 were only 0.5 g/L, suggesting a limited role of the *mar*-regulation in paraben resistance (Table 2).

Among the 23 *E. gergoviae*, methylparaben MICs ranged from 1 to 3.8 g/L, and 7 strains presented an MIC of 3.8 g/L (data not shown). The two strains EG3 and EG7 expressed high esterase activity with complete methylparaben hydrolysis, whereas it did not exceed 15% in other strains (Table 2).[2] No real relationship could be recorded between methylparaben MICs and these variable esterase activities. These results suggest the existence of an additional resistance mechanism.

Characterization of the mar region of E. gergoviae

PCRs were performed on the genomic DNA of the EG7 and EG9 strains. Pairs of primers gave specific amplification products that allowed us to obtain sequences of 1300 bp exhibiting a putative COOH-terminal part of *marC*, *marO*, *marR*, *marA* and *marB* genes. For the two strains studied, an identical sequence was found. The predicted amino acid sequence alignments of MarA and MarR from *E. gergoviae* were compared with *E. coli*, *Enterobacter cloacae*, *E. aerogenes* and *K. pneumoniae* homologues.[4,10] The putative sequence of MarA-*Eg* showed higher amino acid similarity to the *E. aerogenes* sequence (92%) than to the *E. coli* sequence (89%). The amino acid sequences varied in length, with a protein of 131 residues in *E. gergoviae*, a protein of 125 residues for MarA-*Kp* and a protein of 129 amino acids for MarA-*Eco*. The length of the putative sequence of the MarR-*Eg* protein is similar to that for the other MarR. Divergence of amino acid sequences between the different species was higher than that deduced for MarA.

Susceptibility to the efflux pump inhibitor phenylalanine arginine β-naphthylamide (PAβN)

The decrease in MICs of methylparaben obtained with PAβN indicated the presence of an efflux mechanism pumping the biocide out of the strain (Table 2). EG7 was 3 times more resistant than EG4 to methylparaben: this could indicate that esterase expression in EG7 has a protective action towards methylparaben activity. However, in the presence of PAβN, MICs of methylparaben for EG4 and EG7 were similar, indicating that esterase alone is not sufficient to protect the cell.

Potassium efflux measurements

We have observed that addition of propylparaben to *E. coli* cell suspensions leads to potassium leakage.[9] Consequently, the antibacterial activity of propylparaben is caused, at least in part, by the alteration of membrane integrity. Here we observed that *E. gergoviae* membranes were also altered by propylparaben, suggesting a general mechanism for paraben activity.

We compared potassium leakage of propylparaben-susceptible (EG4) and propylparaben-resistant (EG7) *E. gergoviae* strains after addition of propylparaben. The linear rate of K^+ release was about five times higher for EG4 than for EG7 (data not shown). These results indicated that resistance mechanisms detected in EG7, such as efflux pump and esterase expression present in the periplasm, efficiently protected EG7 membrane from propylparaben activity.

Valkova *et al.*[2] have shown that total methylparaben hydrolysis was achieved after 120 min of contact with bacterial cells that expressed high levels of esterase. However, K^+ release appeared during the first 2 min after contact with methylparaben. Thus, esterase alone did not confer a sufficiently rapid and protective action against the toxic effect of methylparaben on membranes. In bacteria, additional efflux of propylparaben generates a synergic effect on resistance, probably by decreasing the periplasmic paraben concentration.

Conclusions

No direct relationship was observed between paraben and antibiotic resistance. This study confirmed that *E. gergoviae* shows natural resistance to parabens by the expression of a PAβN-sensitive efflux mechanism. It is independent of antibiotic efflux, as seen in *Ea* 27, which preserves methylparaben susceptibility, despite overexpression of the AcrAB-TolC efflux pump. Considering that (i) efflux pumps with narrow substrate specificity exist even for antibiotics, as demonstrated for telithromycin in *E. aerogenes*, and that (ii) parabens are quite different from triclosan and salicylate, which selected MDR strains by way of common biocide-antibiotic efflux systems, we propose that parabens are effluxed by a selective pump.[11] Besides, the AaeAB efflux system was recently identified as a specific aromatic carboxylic acid pump in *E. coli*.[12] It is up-regulated by a MarA-independent pathway, and *p*-hydroxybenzoic acid up-regulates the operon *aaeRXAB*.[13] Interestingly, parabens are esters of *p*-hydroxybenzoic acid, so they could be pumped from cells by an analogue of AaeAB in *E. gergoviae*. Although the origin of *E. gergoviae* strains is unknown, this work proposes an explanation for the isolation of *E. gergoviae* in parabens-containing products.

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Transparency declarations

None to declare.

References

1. Soni MG, Burdock GA, Taylor SL *et al.* Safety assessment of propylparaben: a review of the published literature. *Food Chem Toxicol* 2001; **39**: 513–32.

2. Valkova N, Lepine F, Valeanu L *et al. prbA*, a gene coding for an esterase hydrolyzing parabens in *Enterobacter cloacae* and *Enterobacter gergoviae* strains. *J Bacteriol* 2002; **184**: 5011–7.

3. Malléa M, Chevalier J, Bornet C *et al.* Porin alteration and active efflux: two *in vivo* drug resistance strategies used by *Enterobacter aerogenes*. *Microbiology* 1998; **144**: 3003–9.

4. Chollet R, Bollet C, Chevalier J *et al. mar* operon involved in the multidrug resistance in *Enterobacter aerogenes*. *Antimicrob Agents Chemother* 2002; **46**: 1093–7.

5. Davin-Regli A, Monnet D, Saux P *et al.* Molecular epidemiology of *Enterobacter aerogenes* acquisition: one-year prospective study in two intensive care units. *J Clin Microbiol* 1996; **34**: 1474–80.

6. Szybalski W. Microbial selection. I. Gradient plate technique for study of bacterial resistance. *Science* 1952; **116**: 46–8.

7. Ausubel FM, Brent RE, Kingston DD *et al. Current Protocols in Molecular Biology*. New York: John Wiley & Sons, 1995.

8. Nguyen T, Clare B, Guo W *et al.* The effects of parabens on the mechanosensitive channels of *E. coli*. *Eur Biophys J* 2005; **34**: 389–95.

9. Bredin J, Davin-Regli A, Pages J-M. Propyl paraben induces potassium efflux in *Escherichia coli*. *J Antimicrob Chemother* 2005; **55**: 1013–5.

10. Alekshun MN, Levy SB. Regulation of chromosomally mediated multiple antibiotic resistance: the *mar* regulon. *Antimicrob Agents Chemother* 1997; **41**: 2067–75.

11. Chollet R, Chevalier J, Bryskier A *et al.* The AcrAB-TolC pump is involved in macrolide resistance but not in telithromycin efflux in *Enterobacter aerogenes* and *Escherichia coli*. *Antimicrob Agents Chemother* 2004; **48**: 3621–4.

12. Van Dyk TK, Templeton LJ, Cantera KA *et al.* Characterization of the *Escherichia coli* AaeAB efflux pump: a metabolic relief valve? *J Bacteriol* 2004; **186**: 7196–204.

13. Barbosa TM, Levy SB. Differential expression of over 60 chromosomal genes in *Escherichia coli* by constitutive expression of MarA. *J Bacteriol* 2000; **182**: 3467–74.