prbA, a Gene Coding for an Esterase Hydrolyzing Parabens in Enterobacter cloacae and Enterobacter gergoviae Strains

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The new gene *prbA* encodes an esterase responsible for the hydrolysis of the ester bond of parabens in *Enterobacter cloacae* strain EM. This gene is located on the chromosome of strain EM and was cloned by several PCR approaches. The *prbA* gene codes for an immature protein of 533 amino acids, the first 31 of which represent a proposed signal peptide yielding a mature protein of a putative molecular mass of 54.6 kDa. This enzyme presents analogies with other type B carboxylesterases, mainly of eukaryotic origin. The cloning and expression of the *prbA* gene in a strain of *Escherichia coli* previously unable to hydrolyze parabens resulted in the acquisition of a hydrolytic capacity comparable to the original activity of strain EM, along with an increased resistance of the transformed strain to methyl paraben. The presence of homologues of *prbA* was tested in additional ubiquitous bacteria, which may be causative factors in opportunistic infections, including *Enterobacter gergoviae*, *Enterobacter aerogenes*, *Pseudomonas agglomerans*, *E. coli*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*. Among the 41 total strains tested, 2 strains of *E. gergoviae* and 1 strain of *Burkholderia cepacia* were able to degrade almost completely 800 mg of methyl paraben liter^{-1}. Two strains of *E. gergoviae*, named G1 and G12, contained a gene that showed high homology to the *prbA* gene of *E. cloacae* and demonstrated comparable paraben esterase activities. The significant geographical distance between the locations of the isolated *E. cloacae* and *E. gergoviae* strains suggests the possibility of an efficient transfer mechanism of the *prbA* gene, conferring additional resistance to parabens in ubiquitous bacteria that represent a common source of opportunistic infections.

Bacterial hydrolysis of antimicrobial agents used as preservatives in common pharmaceutical, cosmetic, or food products is a significant medical and economic concern. The esters of 4-hydroxybenzoic acid, commonly named parabens, are important commercial preservatives. Parabens are often used by the pharmaceutical, cosmetic, and food industries due to their excellent stability, wide pH range, antimicrobial spectrum of activity, and low toxicity (8, 16, 20). The ability of various species of *Enterobacter*, *Alcaligenes*, and *Pseudomonas* to utilize parabens for growth has been demonstrated (3). Additionally, the resistance to these compounds by the hydrolysis of their ester linkage has been documented for *Cladosporium resinae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (4, 19, 21, 30). Although some of these studies speculated on the role of an esterase in the hydrolysis of parabens, the identification of such an enzyme has not been reported.

Many of the organisms cited in the literature as showing resistance to parabens are bacterial species that may become opportunistic pathogens. Numerous cases of nosocomial infections by various species of *Enterobacter* introduced through contaminated medical products have been reported (2, 24, 27). Recently, we described the *Enterobacter cloacae* strain EM, which was isolated from a contaminated batch of a commercial mineral supplement normally well stabilized with a mixture of 1,700 mg of methyl paraben liter^{-1} (11.2 mM) and 180 mg of

propyl paraben liter^{-1} (1.0 mM). This strain demonstrated a very high resistance to the parabens attributed to the action of an esterase (26). Here, we report the cloning and sequencing of the gene named *prbA* coding for this esterase, which is responsible for the hydrolysis of the parabens, and the presence of close homologues of this enzyme in *Enterobacter gergoviae* strains, which also show a high resistance to parabens.

MATERIALS AND METHODS

Materials. Culture media were obtained as follows: tryptic soy broth (TSB), tryptic soy agar (TSA), tryptone-peptone, and agar were purchased from Difco (Sparks, Md.). Yeast extract, glucose, and sodium dodecyl sulfate were obtained from ICN Biomedicals (Aurora, Ohio). Methyl paraben was obtained from Sigma-Aldrich (St. Louis, Mo.), polyethylene glycol was obtained from A & C American Chemicals (Fair Lawn, N.J.), and agarose was obtained from Life Technologies (Grand Island, N.Y.). DNA was purified from agarose with the Qiaex II gel extraction kit from Qiagen (Mississauga, Ontario, Canada), and plasmid DNA was further purified with the QIAprep spin miniprep kit (Qiagen). All restriction enzymes were purchased from Pharmacia Biotech (Baie d'Urfé, Quebec, Canada). Lysozyme was obtained from Roche (Laval, Quebec, Canada), and proteinase K was obtained from Qiagen. All reagents for PCRs, including *Taq* polymerase, were obtained from Pharmacia Biotech. All reagents for ligation, including T4 DNA ligase, were included in the pGEM-T Easy Vector System I from Promega (Madison, Wis.). Digoxigenin (DIG)-labeled DNA was constructed with the DIG DNA labeling kit from Roche according to the manufacturer's instructions. Nylon membranes were obtained from Roche, and Biomax MR Scientific Imaging Film was obtained from Kodak (Rochester, N.Y.).

Cloning of the *prbA* gene from *E. cloacae* strain EM. The degenerate primers EcoN and EcoPep4 were used to amplify a 300-bp stretch of the N terminus of the esterase of strain EM. The sequence of the primer EcoN (5′-AT(T/C/A)G A(G/A)GGNGTNAA(G/A)AA(T/C)GA-3′) was derived from a stretch of 20 amino acids comprising the N terminus of the protein, as determined by Edman degradation of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis band, and consisting of the amino acids QELSPVVQMSKGTIEGVKND. Be-

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Dradiodurans1    :   tnaqka-PVmVWIHGGsfqmGagsd
Dradiodurans2    :   pnakna-PllVWvHGGfftgGdasl
Dacidovorans     :   atgegpfPVmVWIHGGafsiGgtit
Scoelicolor1     :   p-dggplPVlVWIHGGaytfGssaq
Scoelicolor2     :   pgpgarlPVlVWlHGGaltrGssav
Bacillus BP-23   :   kesshplPVmVWIHGasfvtGsgsl
Bacillus BP-7    :   tpgkn-rPVmVWIHGGtfylGagse
Mtuberculosis    :   epatqplPVmVfIHGGgyilGssat
Aoxydans         :   ldggs-rPVlVWIHGGglltGsgnl
Mpersicae        :   nsagdlmnViVhIHGGgyyfGegil
Celegans         :   cmkrkncsVmivvHGGriltesasa
CONSENSUS        :   -------PVmVWIHGG----G----
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FIG. 1. Conserved amino acid region in type B carboxylesterases. The amino acid sequences of representative carboxylesterases were aligned with the Pileup program: *Deinococcus radiodurans*, GenBank accession no. AAF12163 and AAF09993; *Delftia acidovorans*, BAA76305; *Streptomyces coelicolor*, CAB55678 and CAA22794; *Bacillus* sp. strains BP-23 and BP-7, CAB42083 and CAB93516, respectively; *Mycobacterium tuberculosis*, CAA17259; *Arthrobacter oxydans*, AAA22078; *Myzus persicae*, CAA52649; *Caenorhabditis elegans*, CAB03277. Amino acid numbering refers to the *D. radiodurans* 1 sequence.

cause the N-terminal portion showed significant similarity to type B carboxylesterases, an alignment of the amino acid sequences of representative carboxylesterases revealed a conserved 9-amino-acid region (Fig. 1). The first 8 amino acids of this region (sequence PVMVWIHG) were suitable for the construction of the second primer, EcoPep4, because they contained the codons with minimum degeneracy at the third position necessary to maximize the chances of hybridization with the unknown *prbA* gene. The deduced sequence of the EcoPep4 primer was 5′-CC(A/G)TG(T/G/A)ATCCANACCATNACNGG-3′. Due to the degeneracy of the primers, a touchdown PCR approach was selected, to facilitate the hybridization of degenerate primers to their proper target (5). The amplification conditions were as follows: 80°C for 3 min and 94°C for 5 min (1 cycle); 60°C for 1 min, 72°C for 2 min, and 94°C for 40 s (3 cycles); repeats of the previous parameters at decreasing 2°C increments until 42°C for 3 cycles each; 40°C for 1 min, 72°C for 2 min, and 94°C for 40 s (14 cycles); and 40°C for 1 min, followed by 72°C for 10 min (1 cycle). A 300-bp stretch of DNA thus amplified was purified from agarose, ligated into pGEM-T Easy, and transformed into *E. coli* DH5α cells. Subsequently, the pGEM-T Easy plasmid containing the DNA of interest was purified, and the 300-bp fragment was sequenced.

In order to amplify sections of the chromosome of strain EM containing the entire *prbA* gene, a DNA-DNA hybridization probe was constructed by labeling the purified 300-bp N-terminal region with DIG, yielding a probe of suitable size to ensure specificity. The chromosomal DNA of strain EM was subsequently fragmented by four common restriction enzymes. The fragments generated were transferred by Southern blotting to a hybridization membrane and tested with the 300-bp DIG probe at 65°C. *Eco*RI and *Hin*dIII produced DNA fragments of 3.0 and 2.8 kb, respectively, that hybridized with the probe. They were subsequently self-circularized, and inverse PCR was used to amplify the unknown sequences flanking the 300-bp fragment (25). The selected primers for inverse PCR, L$_1$R and L$_2$F, were constructed from the extremities of the only known portion of the esterase sequence, the 300-bp fragment of the N terminus. These primers were designed to hybridize at the opposite ends of the 300-bp portion of the gene and to direct amplification around the entire self-circularized fragment. The sequence of the reverse primer L$_1$R was 5′-GGTTCGCTTTTAAC GTGCCC-3′, and that of the forward primer L$_2$F was 5′-CACCAAGCTCACT TTGGGTG-3′. The PCR conditions were as follows: 1 cycle of 80°C for 2 min, 94°C for 5 min, and 55°C for 5 min; and 30 cycles of 72°C for 2 min, 94°C for 40 s, and 55°C for 1 min; followed by 72°C for 10 min. The amplified 2.8- and 3.0-kb fragments were subsequently ligated into pGEM-T Easy, and the same procedure described above was used for sequencing the 300-bp fragment.

Expression of the *prbA* gene in *E. coli* DH5α. Two primers were designed based on the complete sequence of the *prbA* gene in order to amplify the gene from the chromosomal DNA of strain EM. The sequence of the forward primer was 5′-AAGGGAAATAATGGAACTAC-3′ (nucleotides 238 to 257 [GenBank accession no. AY077721]). This sequence included 10 nucleotides upstream of the methionine start codon of *prbA*, and the transcription initiation site and promoter for the expression of the gene were those present upstream of the multiple cloning site of pGEM-T Easy. The sequence of the reverse primer was 5′-TACCCCTGCGAACTCATCAC-3′ (nucleotides 1824 to 1843). Due to po-

tential primer instability, the reverse primer ended 3 nucleotides before the stop codon, and the amplified product was ligated in frame with a stop codon in the multiple cloning site of pGEM-T Easy. The PCR amplification protocol was the same as that used above for the amplification of the 2.8-kb and 3.0-kb fragments. The amplified gene was ligated into the pGEM-T Easy vector and transformed into competent *E. coli* DH5α cells. The hydrolysis of 600 mg of methyl paraben liter^{-1} (4.0 mM) in TSB growth medium was monitored at 37°C as previously described (26).

Resistance to parabens and presence of *prbA* homologues in related strains. A series of *Enterobacter* strains were obtained from various sources, many of which were isolated from commercial products stabilized with parabens (Table 1). The MICs were determined by inoculating a culture pregrown in TSB to the stationary phase in TSB containing 200, 400, 800, 1,600, or 3,200 mg of methyl paraben liter^{-1} to an optical density at 600 nm (OD$_{600}$) of 0.05, where an OD of 1.0 corresponded to 6×10^8 *Enterobacter* cells per ml, and incubating the culture for 24 h at 30°C with shaking. OD readings were taken at the moment of inoculation and after 24 h, and the MICs were defined as the minimal amount of methyl paraben that prevented an increase in the OD of the suspension. To determine the efficiency of the strains to degrade parabens, the cells of exponentially growing cultures at an OD of 2.0 were harvested by centrifugation and resuspended in TSB medium containing 800 mg (5.3 mM) of methyl paraben liter^{-1} for a 2-h incubation period at 30°C with shaking. At the beginning and end of the 2-h incubation, aliquots were removed and heated immediately to 80°C for 10 min in order to prevent further enzymatic degradation of the parabens. The amount of parabens remaining in the samples and the appearance of *p*-hydroxybenzoic acid was then analyzed by high-performance liquid chromatography as previously described (26). The presence of homologues of *prbA* in selected strains was determined by touchdown PCR with the primers and conditions specified above.

Nucleotide sequence accession number. The GenBank accession numbers for the complete sequence of *prbA* from *E. cloacae* EM and the partial sequences for *prbA* from *E. gergoviae* G1 and G12 are AY077721, AY077722, and AY077723.

RESULTS

Cloning of the *prbA* gene from strain EM. To amplify an initial segment of the N terminus of the esterase, the degenerate primers EcoN and EcoPep4 were hybridized to both the chromosomal and plasmid DNA of *E. cloacae* strain EM by touchdown PCR. A strong amplification signal of a 300-bp fragment was obtained with the chromosomal DNA of strain EM (Fig. 2A, lane *a*). No corresponding 300-bp fragment was found with the plasmid DNA of strain EM (Fig. 2A, lane *b*). The sequence of the amplified 300-bp fragment from the chromosomal DNA of strain EM contains at each extremity the sequence of the respective primers EcoN and EcoPep4, as well as 92 codons in frame between these sequences, and its deduced amino acid sequence showed homology to other type B carboxylesterases.

Sections of the chromosome of strain EM containing the esterase gene were identified with a DIG-labeled probe constructed from the 300-bp N terminus of the esterase (Fig. 2B). The probe hybridized to fragments of various sizes generated with all four of the selected restriction enzymes. The two fragments generated by *Eco*RI (lane *b* [3.0 kb]) and by *Hin*dIII (lane *c* [2.8 kb]) were targeted for separate extraction and cloning. To extract and amplify a sufficient quantity of the 2.8-kb fragment generated by *Hin*dIII (Fig. 2B, lane *c*), 100 μg of chromosomal DNA was cut with this enzyme, and all of the fragments migrating between 2.5 and 3.0 kb were purified (Fig. 2C). To ensure that the fragment of interest was present, the extracted fragments were transferred to a hybridization membrane by Southern blotting and tested with the 300-bp DIG probe. As shown in Fig. 2C, a fragment of the appropriate size hybridized with the probe, suggesting that the targeted 2.8-kb fragment containing the esterase gene had been extracted. The

TABLE 1. Origin of strains, MICs, and hydrolytic ability

Strain designation	Origin	MIC (mg liter^{-1})[f]	Degradation (%)[g]
E. cloacae			
EM[a]	Mineral supplement	>3,200	99.9
ID 67037[b]	Clinical strain	3,200	12.7
ID 67284[b]	Clinical strain	3,200	12.8
LSPQ 3022	Reference strain	3,200	15.3
LSPQ 3345	Reference strain	3,200	19.2
E. gergoviae			
G1[c]	Eye liner	>3,200	100
G2[c]	Foundation	3,200	10.3
G3[c]	Exfoliating gel	3,200	8.9
G4[c]	Foundation	3,200	8.5
G5[c]	Clinical strain	3,200	7.8
G6[c]	Cream	3,200	10.4
G7[c]	Challenge test	3,200	14.7
G8[c]	Shampoo	3,200	14.4
G9[c]	Cream	3,200	11.4
G10[c]	Shampoo	3,200	12.3
G11[c]	Cream	3,200	16.3
G12[c]	Rinsing material	3,200	97.9
G13[c]	Clinical strain	1,600	15.5
G14[c]	Clinical strain	3,200	20.0
G15[c]	Clinical strain	3,200	22.6
GM[a]	Mineral supplement	3,200	18.7
WL[d]	Syrup	3,200	14.8
LSPQ 3347	Reference strain	3,200	14.0
ATCC 33028	Reference strain	3,200	12.6
ATCC 33426	Reference strain	3,200	14.7
ATCC 33428	Reference strain	3,200	18.1
E. aerogenes			
AE1[c]	Clinical strain	3,200	15.4
AE1[c]	Clinical strain	3,200	12.7
AE1[c]	Clinical strain	3,200	14.7
LSPQ 3811	Reference strain	3,200	15.6
ATCC 13048	Reference strain	3,200	12.9
P. agglomerans			
ID 60635[b]	Clinical strain	3,200	10.1
ID 65518[b]	Clinical strain	3,200	14.4
LSPQ 3353	Reference strain	1,600	14.5
LSPQ 3825	Reference strain	800	13.5
E. coli			
ID 41839[e]	Clinical strain	3,200	9.1
ATCC 25922	Reference strain	1,600	12.6
ATCC 35218	Reference strain	3,200	11.3
B. cepacia IFO 15124	Oil-in-water emulsion	1,600	37.9
P. aeruginosa			
ID 33122[e]	Clinical strain	3,200	12.4
ATCC 33350	Reference strain	1,600	10.2

[a] Provided by a private company in Quebec, Canada.
[b] Provided by the "Laboratoire de Santé Publique" (LSPQ), Quebec, Canada.
[c] Provided by C. Bollet, Faculty of Medicine, INSERM, Marseille, France.
[d] Provided by a private company in Ontario, Canada.
[e] Provided by the Maisonneuve-Rosemont Hospital, Montreal, Canada.
[f] Expressed as the concentration of methyl paraben.
[g] Expressed as the percentage of 800 ppm (5.7 mM) of methyl paraben degraded after 2 h of incubation by an exponentially growing culture.

purified fragments were subsequently self-circularized, and the 2.8-kb fragment was amplified by inverse PCR. As shown in Fig. 2D, a single fragment of the appropriate size expected from a *Hin*dIII cut was amplified. The same procedure, as illustrated in Fig. 2C and D, was used to clone the fragment of 3.0 kb corresponding to the *Eco*RI cut (Fig. 2B, lane *b*), which

also contained the esterase gene. To ensure the accuracy of the sequence and to fill in small gaps created by the cloning procedure, both the 2.8- and 3.0-kb fragments were sequenced on both the forward and reverse strands.

The nucleotide sequence of the esterase gene of *E. cloacae* strain EM, named *prbA* (for paraben A), has been annotated and deposited in the GenBank public database. The coding sequence is composed of 1,602 nucleotides, including the stop codon, constituting an immature protein of 533 amino acids with a theoretical molecular mass of 57.9 kDa (Protein General Sequence Analysis, UK HGMP Resource Centre) (29). The first 31-amino-acid stretch of the protein (nucleotides 248 to 340) is believed to represent a signal peptide, according to the signal peptide prediction tool SignalP v1.1 (Technical University of Denmark), which suggested a cleavage site between amino acids 31 (A) and 32 (Q) (14). Additionally, the prediction tool TMHMM v2.0 (Technical University of Denmark) for transmembrane helices in proteins suggested that a transmembrane helix exists between amino acids 12 (L) and 31 (A), with amino acids 1 to 11 (M—I) inside the cytosol and amino acids 32 to 533 outside the cell membrane (11). After cleavage of the signal peptide, the mature protein would contain 502 amino acids, with a theoretical molecular mass of 54.6 kDa (29). Analysis of the mature amino acid sequence with the prediction tool PredictProtein (Columbia University Bioinformatics Center) detected a carboxylesterase type B signature 2 motif between amino acids 78 and 88 (EDCLYLNVYTP) as well as a carboxylesterase type B serine active site between amino acids 176 and 191 (FGGDNHNVTLFGESAG). Additionally, secondary structure predictions with the same analysis tool suggested a compact globular protein, with 25% α-helices, 20% β-strands, and the remaining 55% as loop (or other) structures (9, 17, 18).

Expression of the *prbA* gene in *E. coli* DH5α. The cloning and expression of the *prbA* gene from *E. cloacae* strain EM into *E. coli* DH5α resulted in the acquisition of a paraben esterase activity comparable to that of the original *E. cloacae* strain (Fig. 3). *E. coli* DH5α containing the *prbA* gene was able to hydrolyze 49% of 600 mg of methyl paraben liter^{-1} (4.0 mM) within 30 min of inoculation and 97% after 90 min. In comparison, strain *E. cloacae* EM hydrolyzed 93% of methyl paraben after 30 min and 99.6% after 90 min (Fig. 3). The slightly slower rate of hydrolysis in *E. coli* compared to that in *E. cloacae* may be due to differences in expression of the protein between the two strains. No paraben esterase activity was present in *E. coli* DH5α transformed with the pGEM-T Easy vector alone as shown by the constant amount of methyl paraben remaining in solution and by the lack of production of *p*-hydroxybenzoic acid (Fig. 3), and similarly, no paraben esterase activity was found in *E. coli* DH5α cells alone (results not shown). Additionally, the MIC for methyl paraben increased from 3,200 ppm for *E. coli* DH5α cells without the *prbA* gene to >3,200 ppm for DH5α cells containing *prbA*. This increased resistance caused by the *prbA* gene is better illustrated by the large increase in the OD of the *prbA*-containing strain, which increased from 0.174 to 1.64 after a 24-h incubation, in comparison with the unmodified *E. coli* DH5α strain that presented only a small OD increase from 0.155 to 0.207 during the same period.
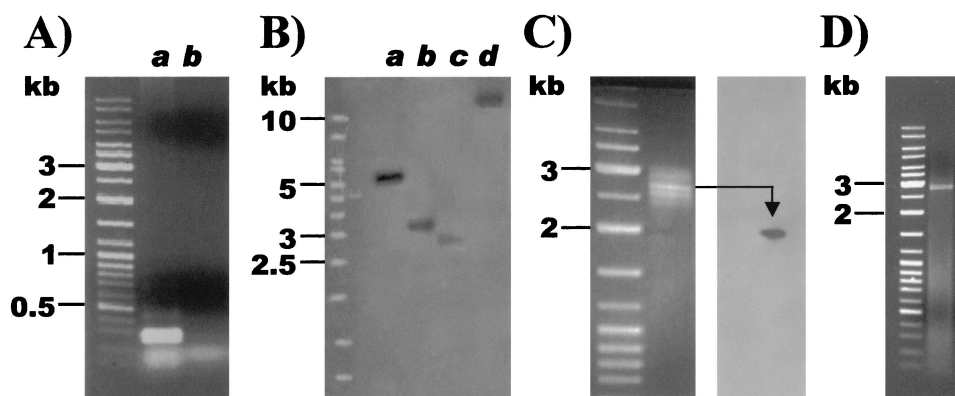
FIG. 2. Cloning of the *prbA* gene. (A) Amplification of the 300-bp N-terminus region of strain EM by touchdown PCR. Lanes: *a*, chromosomal DNA; *b*, plasmid DNA. (B) Hybridization of chromosomal fragments of strain EM generated by *Bam*HI (lane *a*), *Eco*RI (lane *b*), *Hin*dIII (lane *c*), *Pst*I (lane *d*) with the digoxigenin-labeled N terminus of *prbA*. (C) Extraction of the chromosomal fragments between 2.5 and 3.0 kb generated by *Hin*dIII and hybridization with the digoxigenin probe. (D) Amplification of the 2.8-kb chromosomal fragment generated by *Hin*dIII containing the *prbA* gene by inverse PCR.

Resistance to parabens in related strains. The resistance to parabens of other strains closely related to *E. cloacae* EM was measured with 3 other *E. cloacae* strains, 21 *E. gergoviae* strains, 5 *E. aerogenes* strains, and 4 *P. agglomerans* strains obtained from various commercial or clinical sources or from bacterial collections (Table 1). Three *E. coli* and two *Ps. aeruginosa* strains were selected as ubiquitous bacteria, which may represent a health risk, and finally a strain of *B. cepacia* previously known to hydrolyze parabens was added (21). These strains were tested for their resistance to the most soluble of the parabens, methyl paraben, in liquid aerated culture. Their resistance was expressed as the MIC of methyl paraben (Table 1). Although the MIC does not provide indications of the mechanism of resistance, it shows the ability of the strains to grow in the presence of this preservative. As seen in Table 1, virtually all strains (except *P. agglomerans* LSPQ 3825) showed a high tolerance to this agent, with MICs of methyl paraben of 1,600 mg liter^{-1} (10.5 mM) or greater. In fact, the MIC for the majority of the strains, of either commercial or clinical origin, was 3,200 mg liter^{-1}, regardless of the lack of previous known exposure to this biocide. The MIC for *E. cloacae* strain EM, containing the *prbA* gene, predictably was very high, greater than 3,200 mg liter^{-1}, which is near the limit of solubility of this paraben and exceeds the concentration normally added to commercial products, which is generally less than 2000 mg liter^{-1} (7, 16). Interestingly, the MIC for a strain of *E. gergoviae* (G1) was also >3,200 mg liter^{-1}, comparable to that of strain EM.

The ability of these strains to degrade methyl paraben was then tested as a potential indication of the presence of a gene similar to *prbA*. Degradation was measured by selecting a concentration of methyl paraben at which all the strains could grow (800 mg liter^{-1}) and incubating exponentially growing cells at this concentration (Table 1). Despite their high resistance, as indicated by the MICs, 37 of the 41 strains showed a poor capacity to degrade the paraben, ranging from 8 to 23% degradation, with a median value of 13.5%. Among the remaining four strains, *E. cloacae* EM was able to hydrolyze 99.9% of the paraben, while *E. gergoviae* strain G1, which had shown a comparable MIC, degraded 100% of the paraben.

Another *E. gergoviae* strain (G12) also showed a significant degradation capacity, removing 97.9% of the paraben, while the *B. cepacia* strain previously shown to have hydrolytic capacity (21) was able to degrade only 37.9% of the paraben. The ability of strains EM, G1, and G12 to hydrolyze the ester bond of methyl paraben was further confirmed by monitoring the appearance of the degradation product (*p*-hydroxybenzoic acid) concurrent with the disappearance of methyl paraben (Fig. 4). All three strains showed a high paraben esterase activity. Strain EM hydrolyzed 96% of the methyl paraben within 15 min of inoculation, while in the same period of time, strain G1 hydrolyzed 15%, and strain G12 hydrolyzed 3.5% of the paraben. After 120 min, strain EM had completely hydrolyzed 800 mg of methyl paraben liter^{-1}, while strain G1 hy-
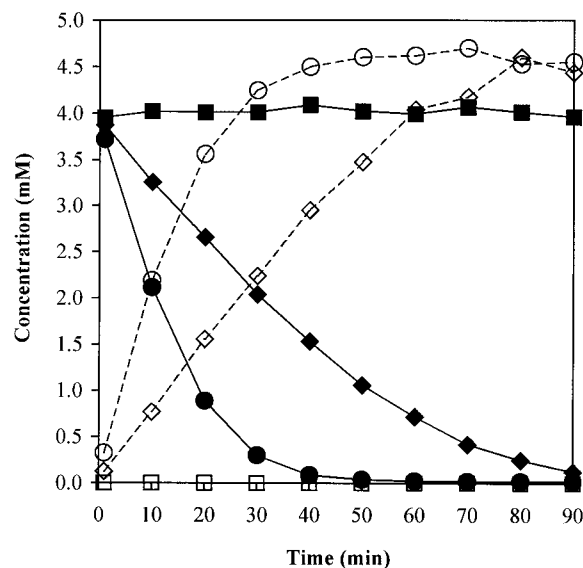

FIG. 3. Hydrolysis of 600 mg of methyl paraben liter−1 (4.0 mM) (solid lines) and production of *p*-hydroxybenzoic acid (dotted lines) by *E. coli* DH5α containing the *prbA* gene cloned in the pGEM-T Easy vector (◆ and ◇), by the *E. cloacae* strain EM (● and ○), and by *E. coli* DH5α containing the pGEM-T Easy vector alone (■ and □).
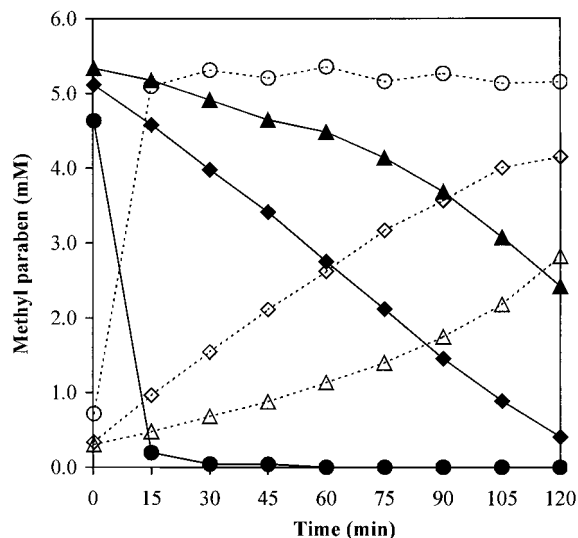
FIG. 4. Paraben esterase activity in strains *E. cloacae* EM (● and ○) and *E. gergoviae* G1 (◆ and ◇) and G12 (△ and ▲). The amount of the degradation product *p*-hydroxybenzoic acid produced (dotted lines) is consistent with the amount of methyl paraben concurrently degraded (solid lines).

drolyzed 92%, and strain G12 hydrolyzed 55% of the paraben. The *B. cepacia* IFO strain did not substantially hydrolyze the paraben within these time periods. Therefore, in addition to strain EM, the *E. gergoviae* strains G1 and G12 were selected as potentially harboring a gene of similar function to *prbA*.

Presence of homologues of *prbA* in the other strains. To determine if a homologue of the gene *prbA* was present in *E. gergoviae* strains G1 and G12 or *B. cepacia* IFO, a touchdown PCR approach was chosen to amplify the 300-bp N-terminus portion previously amplified in strain EM. The same primers as used with strain EM were able to anneal to the chromosomal DNA of strains G1 and G12 and showed an amplification signal of similar intensity that migrates accordingly to its expected size of 300 bp. This finding confirmed that a homologous esterase was present in *E. gergoviae* strains G1 and G12 and that their N termini were sufficiently similar to that of *prbA* from strain EM to hybridize to the same primers. No amplification was observed with the chromosomal or plasmid DNA of *B. cepacia* strain IFO, suggesting that the esterase that hydrolyzes the parabens in this strain was considerably different from those of the hydrolases in the *Enterobacter* strains. No significant amplification was detected on plasmid DNA of either of the *E. gergoviae* strains, suggesting that, as in the case of *E. cloacae*, the esterase is of chromosomal origin.

An alignment of the sequences of the N termini of the esterases present in *E. gergoviae* G1 and G12 and *E. cloacae* EM showed that the sequences of the N termini are virtually identical between the three strains, showing only 11 nucleotide substitutions, the majority of which are in the wobble position and do not affect the identity of the amino acid. Analysis of the nucleotide sequences with the alignment software BLAST 2 Sequences (National Center for Biotechnology Information, Bethesda, Md.) showed that the sequences of the G1 and G12 gene fragments were 97 and 96% identical to that of EM,

respectively, while the sequences of G1 and G12 were 98% identical to each other (22).

DISCUSSION

Previously, we demonstrated that strain EM was able to rapidly hydrolyze the ester bond of parabens by the action of an esterase (26). The gene corresponding to this enzyme, located on the chromosome of strain EM, was named *prbA* and coded for a mature enzyme of 54.6 kDa. A putative signal peptide was found, suggesting that the enzyme may be targeted to the membrane and released into the periplasmic space after cleavage of the signal peptide. This is consistent with previous studies on the ability of whole cells and cell extracts of strain EM to hydrolyze the parabens, which indicated an intracellular or periplasmic location (26) and is consistent with the sequence of the N terminus of the mature protein determined by Edman degradation. Introduction of the *prbA* gene into an *E. coli* strain that did not initially have a paraben-degrading capability resulted in the hydrolysis of the paraben and the concurrent production of *p*-hydroxybenzoic acid and an increased MIC for methyl paraben. This transfer confirms that *prbA* encodes for a paraben esterase in *E. cloacae* strain EM. Recently, the salicylate esterase gene *salE* in *Acinetobacter* was shown to be part of an operon regulating salicylate catabolism (10). However, this gene participates in channeling salicylate esters into the β-ketoadipate pathway through a salicylate hydroxylase, while the *p*-hydroxybenzoic acid generated from parabens by *prbA* is not channeled through this pathway by *E. cloacae* EM, but instead is converted into phenol (26).

As observed previously (26), strain *E. cloacae* EM showed a higher resistance to parabens than the other *E. cloacae* strains (Table 1). This increase in resistance could be attributed to the ability to degrade parabens provided by the esterase encoded by the *prbA* gene. A similar increase in paraben resistance in strain *E. gergoviae* G1 could also be attributed to the presence of a homologue of *prbA*, allowing it to completely hydrolyze the same amount of methyl paraben as the *E. cloacae* strain EM in 2 h. The second strain of *E. gergoviae* (G12) also contained a homologue of *prbA*. However, the MIC for the second strain was similar to those for the other *E. gergoviae* strains, with the exception of strain G1. The fact that it did not show an increased resistance to paraben could be explained by the fact that its hydrolytic activity was smaller than those of EM and G1 (Fig. 4). Previous reports have indicated a MIC of methyl paraben for *E. cloacae* of 1,000 mg liter^{-1} (8). We report MICs of methyl paraben approximately threefold higher for almost all *Enterobacter* strains tested. However, the MICs in this report were determined in liquid aerated media, while previous determinations were made on solid media and with a lower inoculum size (10^6 to 10^7 cells/ml versus approximately 3×10^7 cells/ml in this report). In the present work, the majority of the enterobacterial strains tested demonstrated comparably high MICs without the accompanying hydrolysis of the paraben, suggesting an efficient mechanism of resistance to these compounds. A well-documented method of resistance in *E. coli* is the efflux of a variety of structurally distinct biocides through membrane proteins of a wide specificity serving as pumps (15). Although less well investigated, there are indications that similar efflux mechanisms exist in *Enterobacteriaceae* (6, 12), rais-

ing the possibility that the remaining species tested may have resistance to the parabens by a mechanism of efflux. Nevertheless, the increased resistance to methyl paraben of strains EM and G1 and of the *E. coli* DH5α strain containing the *prbA* gene shows that a high hydrolytic activity increases the resistance to methyl paraben, as shown by the increases in the MICs for these bacteria.

Analysis of the *prbA* gene from *E. cloacae* EM with the nucleotide database by using the nucleotide-nucleotide BLAST program showed a conserved stretch of 23 nucleotides (positions 1679 to 1701) corresponding to the sequence TACTGG ACCAACTTTGCCAAAAC, translating to the amino acids YWTNFAK (positions 447 to 453), near the protein's C terminus (1). These nucleotides show 100% identity to a number of eukaryotic esterases. These esterases were predominantly of human origin (*Homo sapiens*) and represent either bile salt-dependent lipases or cholesterol esterases, although several others were carboxyl lipases or cholesterol esterases from the rat (*Rattus norvegicus*), the gorilla (*Gorilla gorilla*), or the rabbit (*Oryctolagus cuniculus*). A similar alignment of the amino acid sequence of PrbA by using protein-protein BLAST showed significant homology (greater than 200 bits) to approximately 100 other esterases. The great majority of the corresponding proteins were carboxylesterases of eukaryotic origin, mostly of rat, mouse, and human origin, as well as others from rabbit, hamster, mallard, monkey, dog, bovine, or pig origin. However, a few esterases were of prokaryotic origin, among them, carboxylesterases from *Deinococcus radiodurans*, *Salmonella* sp., *Bacillus subtilis*, and *Delftia acidovorans*. Some of these esterases showed very high homology scores to PrbA. Among the top 10 homologous esterases found by the BLAST search, four were of prokaryotic origin, including a type B carboxylesterase from *D. radiodurans*, a polyurethane esterase from *D. acidovorans* and two esterases from *Salmonella* sp. Additionally, the two proteins with the greatest amino acid identities to PrbA were the polyurethane esterase from *D. acidovorans* (38%) and the carboxylesterase from *D. radiodurans* (37%).

The sequences of the N termini of the esterases present in strains *E. gergoviae* G1 and G12 were almost identical to each other and to the esterase of strain EM. The sequences of strains G1 and G12 are closer to each other than to strain EM, the two former strains being from the same species and from a similar geographical location. Although the amino acid sequences of *prbA* between strains G1 and G12 are 100% identical, the differences in certain wobble positions of their nucleotide sequences suggest a certain evolution of the protein within the species. However, the minimal divergence between the sequences of the N termini of the esterases of both *E. gergoviae* strains, isolated in France, and of *E. cloacae* EM, originating in North America, suggests that the acquisition of this gene by either species was recent in evolutionary terms.

The analysis of the sequences of PrbA from *E. cloacae* EM and those of *E. gergoviae* G1 and G12 seems to indicate greater homology to a number of esterases of eukaryotic origin from animals that are normally hosts to enteric bacteria. Additionally, many of the homologous esterases were bile salt dependent or similarly localized to the intestinal tract of the host animals. A precedent exists for a eukaryotic-like lipase discovered in *P. aeruginosa*, which is postulated to have been acquired by horizontal transfer (28), and it is known that bile

salts possess the ability to disrupt bacterial membranes (13, 23), hence possibly facilitating the acquisition of extraneous DNA. It is possible that the paraben-hydrolyzing esterase PrbA was acquired by *Enterobacter* sp. through horizontal transfer from a eukaryotic host. However, the significant homologies present between PrbA and a few prokaryotic esterases, which in some cases are from nonenteric species, indicate either convergent evolution or, alternatively, a more distant origin for the PrbA esterase. Because *Enterobacter* species are ubiquitous in nature, the presence of certain strains containing the esterase able to hydrolyze parabens raises health concerns. Such strains may be introduced in commercial products from the raw materials used during their preparation and thus contaminate and remain viable in the finished product. Under the appropriate settings, such as in persons with weakened immune systems or in hospital wards, these otherwise innocuous strains might become opportunistic pathogens of an infectious nature.

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