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Caractérisation de l'estérase PrbA conférant une résistance accrue aux parabènes chez *Enterobacter cloacae*

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Résumé

Les esters de l'acide 4-hydroxybenzoïque, aussi dénommés parabènes, sont des agents de préservation antibactériens communément utilisés par plusieurs industries pharmaceutiques, alimentaires et cosmétiques. Il existe pourtant des bactéries capables de dégrader ces agents de conservation, et ainsi de poser un danger de contamination dans des produits commerciaux. Une telle souche d'*Enterobacter cloacae*, nommée EM, a été isolée d'un supplément minéral diététique d'origine commerciale normalement bien stabilisé aux parabènes. Cette souche est hautement résistante aux parabènes et démontre des concentrations minimales inhibitrices approximativement doubles de celles citées dans la littérature. Cette souche est capable de dégrader les parabènes en acide 4-hydroxybenzoïque par l'action d'une estérase et de transformer complétement l'acide 4-hydroxybenzoïque ainsi produit en phénol. Le gène codant pour cette estérase a été cloné par diverses approches de PCR et d'hybridation et a été nommé *prbA*. Ce gène est situé sur l'ADN chromosomique de la souche EM et code pour une enzyme mature de 54.6 kDa contenant un peptide signal, la localisant ainsi à l'espace périplasmique. Le transfert du gène *prbA* à une souche d'*Escherichia coli* sensible aux parabènes a résulté en l'acquisition d'une résistance aux parabènes semblable à celle de la souche EM. L'estérase PrbA présente plusieurs homologies avec des carboxylestérases de type B d'origine eucaryotique aussi bien que procaryotique. Il a été déterminé que deux souches d'*Enterobacter gergoviae* capables d'hydrolyser les parabènes en acide 4-hydroxybenzoïque contenaient aussi un gène dont le N-terminal était à plus de 95% identique à celui de *prbA*. L'estérase PrbA a été purifiée par diverses chromatographies sur colonne. Le poids moléculaire de l'estérase mature a été estimé à 54 kDa par SDS-PAGE et a été mesuré à 54 619 ± 1 Da par spectrométrie de masse. L'esterase PrbA est fortement active envers les parabènes avec des substituants méthyle jusqu'à butyle avec une préférence envers les parabènes à courte chaîne alkyle. PrbA présente une faible activité envers l'acétate de *p*-nitrophényle. L'estérase est aussi capable d'hydrolyser des analogues structurels des parabènes, notamment le 3-hydroxybenzoate de méthyle, le 4-

aminobenzoate de méthyle et le vanillate de méthyle. Les conditions optimales d'activité estérasique ont été déterminées à 31°C et à pH 7.0. L'estérase PrbA est capable de transestérifier le méthyle ou le propyle parabène avec une série d'alcools allant du méthanol au *n*-butanol avec des taux de rendement de 64% avec 5% de méthanol en moins de deux heures. L'activité de l'enzyme est partiellement inhibée par des inhibiteurs modifiant spécifiquement les histidines tels que le 1-chloro-3-tosylamido-4-phényle-2-butanone (TPCK) et le 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), et complètement inactivée par le diéthylpyrocarbonate (DEPC), indiquant la participation possible d'une histidine au site catalytique. L'enzyme est aussi totalement inhibée par le diisopropylfluorophosphate (DFP), un composé modifiant spécifiquement les sérines. Il a été déterminé qu'une seule molécule de DFP ajoutée à l'enzyme était suffisante pour inhiber totalement l'activité. Des analyses des fragments tryptiques obtenus de l'enzyme modifiée au DFP ont démontré que le site d'attachement du DFP était la sérine 189, indiquant ainsi la position de la sérine catalytique.

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Liste des abbréviations

API : Analytical Profile Index

ATCC : American Type Culture Collection

BSTFA : Bis-trimethylsilyltrifluoroacetamide

CID : Collision-induced dissociation

CMI : Concentrations minimales inhibitrices

DEPC : Diéthylpyrocarbonate

DFP : Diisopropylfluorophosphate

DIG : Digoxigenin

LSPQ : Laboratoire de Santé Publique du Québec

MBCs : Minimum bactericidal concentrations

MICs : Minimum inhibitory concentrations

OD : Optical density

TLCK : 1-Chloro-3-tosylamido-7-amino-2-heptanone

TPCK : 1-Chloro-3-tosylamido-4-phényle-2-butanone

TSA : Tryptic Soy Agar

TSB : Tryptic Soy Broth

Introduction

Les parabènes sont des esters de l'acide 4-hydroxybenzoïque et représentent un groupe d'agents antimicrobiens. Les esters méthylique, éthylique, propylique et butylique sont les plus communément utilisés commercialement, mais l'ester benzylique est aussi hautement actif (Gottfried, 1962; Haag et Loncrini, 1984). Les parabènes ont originalement été introduits sur le marché après des études portant sur la découverte d'agents de préservation pouvant remplacer les dérivés de l'acide salicylique et benzoïque dans des préparations alimentaires et pharmaceutiques. Ces études ont testé les propriétés antimicrobiennes de plusieurs phénols et acides benzoïques substitués envers les moisissures, levures et bactéries (Cavill et Vincent, 1947; Haag et Loncrini, 1984; Sabalitschka, 1930). Les parabènes sont plus avantageux que les dérivés de l'acide salicylique et benzoïque, puisqu'ils sont actifs entre des valeurs de pH étendues (pH 4 – 8), tandis que l'efficacité des dérivés de ces deux acides est limitée aux pH acides (Haag et Loncrini, 1984). Les parabènes ont aussi plusieurs autres avantages en tant qu'agents de préservation. Ils sont sans couleur, sans odeur, non-volatiles et stables. Ils sont actifs contre un spectre étendu de micro-organismes et sont peu dispendieux aux concentrations utilisées. Les parabènes ont également démontré une faible toxicité aiguë et chronique, et présentent un faible risque de carcinogénicité et de tératogénicité (Haag et Loncrini, 1984).

La structure chimique des parabènes est en relation avec leur activité antimicrobienne. Si la fonction ester de la molécule est enlevée, l'acide 4-hydroxybenzoïque résultant ne possède plus qu'une faible activité antimicrobienne, dans certains cas aussi faible qu'un dixième de l'activité de l'ester méthylique (Cavill et Vincent, 1947; Gottfried, 1962). Alternativement, si le groupement phénolique de la molécule est enlevé ou est placé à une autre position de l'anneau phényle, la molécule résultante, par exemple le salicylate de méthyle, a une activité significativement plus faible (Gottfried, 1962). Pour les parabènes, l'activité antimicrobienne augmente avec

une augmentation de la longueur de la chaîne alkyle. Une relation linéaire existe entre l'activité antimicrobienne et la longueur de la chaîne alkyle des esters du méthyle jusqu'à l'octyle parabène (Russell *et al.*, 1985). Il a aussi été démontré que les esters contenant des groupements branchés sont généralement moins efficaces que les isomères correspondants avec une chaîne alkyle linéaire (Cavill et Vincent, 1947). La solubilité des parabènes décroît avec une augmentation du poids moléculaire de la chaîne alkyle, créant une limitation de l'utilisation de parabènes de haut poids moléculaire en milieux aqueux (Haag et Loncrini, 1984). L'efficacité antimicrobienne des parabènes dans une formulation peut être augmentée en combinant deux parabènes. L'addition de leurs concentrations entraîne ainsi un effet bactéricide cumulatif, alors que leur utilisation séparée aux mêmes concentrations aurait seulement un effet inhibiteur sur la croissance (Gottfried, 1962; Gilliland *et al.*, 1992).

Les parabènes ont plusieurs applications en tant qu'agents antimicrobiens dans les industries cosmétiques, alimentaires et pharmaceutiques. Les esters méthylique ou éthylique sont communément ajoutés à des préparations dermatologiques aqueuses et à des solutions ophtalmiques et parentérales. L'ester propylique peut être utilisé dans des préparations dermatologiques lipoïdales, ou en combinaison avec l'ester méthylique dans des systèmes aqueux. Les esters butylique ou benzylique ont des applications dans des formulations cosmétiques ou pharmaceutiques lipoïdales (Gottfried, 1962). Dans des formulations aqueuses, le méthyle ou propyle parabène peuvent être combinés dans les proportions suivantes: 0.026% et 0.014%, 0.07% et 0.03%, 0.10% et 0.01%, ou 0.18% et 0.02%, respectivement (Gottfried, 1962). Dans des formulations cosmétiques, les parabènes sont souvent utilisés à des concentrations de 0.1 – 0.8% (Haag et Loncrini, 1984). Une étude de 215 produits cosmétiques a démontré que 99% des cosmétiques pour application et 77% des produits pour rinçage contenaient un ou plusieurs parabènes. Parmi les produits contenant des parabènes, 98% contenaient du méthyle parabène, 32% contenaient de l'éthyle parabène et 38% contenaient du propyle parabène, tandis que les butyle et benzyle parabènes étaient présents dans 16% des produits (Rastogi *et al.*, 1995).

Les parabènes sont des agents efficaces contre une variété de micro-organismes. Ils sont généralement plus actifs contre les moisissures et levures que contre les bactéries et sont généralement plus efficaces contre les bactéries Gram-positif que les bactéries Gram-négatif (Haag et Loncrini, 1984). Les parabènes sont aussi efficaces contre la germination et croissance des spores bactériennes (Russell, 1991). Des études sur milieu solide ont établi les concentrations minimales inhibitrices (CMI) contre certains micro-organismes comme étant 2000 ppm (0.2%) de méthyle parabène ou 500 ppm (0.05%) de propyle parabène envers *Bacillus subtilis*, *Staphylococcus aureus* ou *Escherichia coli*; 1000 ppm (0.1%) de méthyle parabène ou 500 ppm de propyle parabène envers *Serratia marcescens*, *Enterobacter cloacae* ou *Klebsiella pneumoniae* et 1000 ppm de méthyle parabène ou 250 ppm (0.025%) de propyle parabène envers *Candida albicans* ou *Aspergillus niger* (Haag et Loncrini, 1984). Des études sur des gélatines communément utilisées comme émulsifiants alimentaires comestibles ont démontré qu'une concentration de 0.15% de l'éthyle, propyle ou butyle parabènes inhibait la croissance de micro-organismes typiques, tandis que cette même concentration de méthyle parabène n'avait qu'un effet partiellement inhibiteur (Aalto *et al.*, 1953). De plus, il a été demontré que les mêmes concentrations intra-cellulaires de méthyle, éthyle ou propyle parabènes étaient requises pour causer une diminution de 50% du taux de croissance d'*E. coli*, suggérant que leur différentes activitiés antimicrobiennes pourraient être expliquées par des différences dans leur taux d'absorption par la cellule (Lang et Rye, 1972; Lang et Rye, 1973).

Le mécanisme d'action et la cible cellulaire des parabènes n'ont pas été définitivement établis, mais plusieurs études suggèrent que les parabènes pourraient avoir des sites d'action multiples à travers la cellule. Les premiers mécanismes proposés suggéraient que la membrane cytoplasmique pourrait être le site d'action à cause de la nature lipophile des parabènes (Freese et Levin, 1978; Wedderbern, 1964; Wyss, 1948). Des études sur *S. marcescens* avec la série allant du méthyle au butyle parabène ont démontré qu'un effet létal était accompagné par une fuite des composants intra-cellulaires, un phénomène généralement dû à des dommages membranaires. La fuite de composants intra-cellulaires était généralement plus étendue chez les bactéries traitées

avec des esters plus hydrophobes, tels que les propyle ou butyle parabènes. L'addition d'un stabilisateur osmotique tel que le sucrose n'a pas affecté le taux de fuite, suggérant que l'effet principal des parabènes est d'endommager la membrane cytoplasmique et non la paroi cellulaire (Furr et Russell, 1972b). De plus, des études sur des formes bactériennes de *Salmonella typhimurium* avec des parois cellulaires défectueuses laissant des phospholipides exposés ont démontré que les parabènes n'induisaient pas la lyse cellulaire, suggérant qu'ils ne causaient pas de désintégration extrême de la membrane cytoplasmique (Furr et Russell, 1972a).

Des études supplémentaires ont été conduites sur des cellules d'*E. coli* ou *S. typhimurium* contenant des parois cellulaires défectueuses laissant des phospholipides exposés à la surface de la cellule. Il a été démontré que les cellules avec la paroi cellulaire la plus dénudée étaient les plus sensibles aux parabènes, et que le butyle parabène était le plus efficace, supportant l'hypothèse que la cible des parabènes est la membrane cellulaire (Russell *et al.*, 1985; Russell *et al.*, 1987; Russell et Furr, 1986; Russell et Gould, 1988). Des études sur l'absorption des propyle et butyle parabènes par des parois cellulaires seules de *S. marcescens* ont démontré une absorption moindre que par des cellules entières, suggérant que les esters diffusent à travers la paroi cellulaire jusqu'à la membrane (Furr et Russell, 1972c). D'autres études ont montré une corrélation entre l'augmentation de l'activité antimicrobienne des parabènes et une augmentation de leur lipophilie, supportant l'hypothèse d'un mécanisme associé avec une solubilisation dans la membrane cellulaire (Eklund, 1980; Fukahori *et al.*, 1996; Hansch *et al.*, 1972).

Un autre mécanisme proposé d'action des parabènes suggère qu'en désorganisant la structure membranaire, ils interfèrent avec l'absorption d'autres substances essentielles à la cellule (Sheu et Freese, 1972; Sheu *et al.*, 1972). Une étude sur des cellules entières ou des vésicules membranaires d'*E. coli* ou de *B. subtilis* a démontré que les parabènes inhibent l'absorption de plusieurs acides aminés, tels que l'alanine, la sérine et la phénylalanine (Eklund, 1980; Freese *et al.*, 1973). Une tendance parallèle a été établie entre l'inhibition de l'absorption d'acides aminés et l'inhibition de croissance causés par les parabènes, suggérant un autre mécanisme d'action des parabènes (Eklund, 1980). Une

étude sur l'absorption de la proline, du glucose et de l'acétate par des cellules d'*E. coli* a aussi démontré une inhibition du transport membranaire causé par les parabènes. Il a été démontré que les parabènes inhibaient l'absorption de ces trois substances, qui sont transportées à l'intérieur de la cellule par trois mécanismes différents. Ces résultats indiquent aussi que les parabènes agissent de façon à fluidiser la membrane et causent une inhibition non-spécifique de l'absorption de substances essentielles (Tatsuguchi *et al.*, 1991).

L'effet des méthyle et butyle parabènes sur la force protomotrice chez des vésicules d'*E. coli* a aussi été étudié. Il a été démontré que les parabènes inhibaient sélectivement un composant de la force protomotrice, la différence de pH, mais non le deuxième composant, qui est le potentiel membranaire $\Delta\psi$ (Eklund, 1985). Ces résultats suggèrent que les parabènes pourraient sélectivement inhiber la perméabilité de la membrane aux protons sans altérer sa perméabilité envers d'autres molécules chargées. Pourtant, les auteurs spéculent que la neutralisation de la force protomotrice ne peut pas être le seul mécanisme d'action des parabènes puisqu'une élimination de la différence de pH seulement est insuffisante pour arrêter le transport membranaire (Eklund, 1985; Ramos et Kaback, 1977). Une synthèse de ces études pourrait suggérer que les parabènes agissent en premier lieu sur la membrane cellulaire, en désordonnant les phospholipides de la membrane. Une telle désorganisation pourrait entraîner par la suite des effets nocifs sur la cellule, car des fonctions essentielles de la membranaire, telles que le transport de substances ou le gradient de protons, seraient inhibées.

Les effets des parabènes sur la synthèse de l'ADN et l'ARN ont aussi été évalués. Une étude sur des cellules de *S. marcescens* a démontré qu'aucun changement de l'ADN ou ARN intra-cellulaire ne se produisait après traitement avec chacun des parabènes de la série méthyle jusqu'au butyle (Furr et Russell, 1972b). Par contre, une autre étude sur les effets des parabènes sur des cellules d'*E. coli* et *B. subtilis* a démontré que la synthèse de l'ADN et de l'ARN était inhibée à des concentrations approximativement doubles de celles requises pour inhiber la croissance (Nes et Eklund, 1983). Une étude similaire a examiné les effets des parabènes sur la synthèse de protéines dans des systèmes sans

cellules de *B. subtilis* et d'*E. coli* et a démontré que la synthèse de protéines chez *B. subtilis* était significativement affectée par les parabènes, tandis que la synthèse protéique chez *E. coli* était significativement moins affectée (Nes et Eklund. 1983).

D'autres hypothèses ont suggéré que l'effet bactériostatique des parabènes est dû à une dénaturation des protéines cytoplasmiques ou à des réactions compétitives avec des co-enzymes (Haag et Loncrini, 1984; Shiralkar et Rege, 1978). Une étude sur les enzymes cytoplasmiques arginine désaminase, carbamate kinase et uréase dans les cellules perméabilisées de divers streptocoques ou dans leur forme purifiée a démontré qu'elles étaient irréversiblement inactivées par le butyle parabène (Ma *et al.*, 1999). Des études chez *Streptococcus mutans* ont démontré que les parabènes inhibaient irréversiblement la glycolyse, avec une efficacité augmentée pour le butyle parabène relativement au méthyle parabène. Additionellement, une inhibition réversible de la F-ATPase chez *S. mutans*, *Streptococcus rattus, Streptococcus sanguis* et *E. coli* par le butyle parabène a été documentée (Ma et Marquis, 1996).

La résistance des micro-organismes envers des antiseptiques ou désinfectants a été moins clairement définie que les résistances envers les antibiotiques. Selon Russell, une souche résistante est une souche non-susceptible à une concentration d'antiseptique ou de désinfectant utilisée en pratique (Russell *et al.*, 1986). Il existe plusieurs cas documentés dans la littérature de micro-organismes résistants aux parabènes. Dans tous les cas où le mécanisme de résistance a été étudié, il a été démontré qu'il s'agissait d'un mécanisme de dégradation de parabènes. Une des premières études sur la résistance aux parabènes a démontré l'existence de plusieurs souches provenant de quelques habitats naturels capables de survivre et de dégrader 0.01% de parabènes sur un milieu de sels minéraux. Par exemple, les micro-organismes résistants isolés d'un extrait de sol incluaient des espèces d'*Alcaligenes*, *Pseudomonas* et *Bacillus*. Des micro-organismes résistants ont été isolés d'eau de fossé, de pluie ou de rivière et incluaient des espèces d'*Alcaligenes*, *Aeromonas* et *Pseudomonas* tandis que des effluents d'une usine contenaient aussi des espèces résistantes d'*Actinobacillus* et d'*Enterobacter* (Beveridge et Hart, 1970).

Certaines études ont rapporté l'isolement d'espèces bactériennes spécifiques résistantes à des concentrations de parabènes communément utilisées, et quelques études ont identifié le mécanisme de résistance. Une souche de *Cladosporium resinae* a été isolée d'une suspension pharmaceutique contenant 0.2% de méthyle parabène. La résistance de cette souche a été attribuée à un mécanisme d'hydrolyse du parabène pour produire de l'acide 4-hydroxybenzoïque et l'alcool correspondant (Sokoloski *et al.*, 1962). Une acyl estérase purifiée d'une souche d'*Aspergillus flavus* était capable d'hydrolyser les méthyle et éthyle parabènes parmi une variété d'autres substrats testés (Child *et al.*, 1971). Une souche de *Pseudomonas* (maintenant *Burkholderia*) *cepacia* isolée d'une émulsion aqueuse d'huile contenant du méthyle et du propyle parabène était capable d'hydrolyser les deux parabènes et d'utiliser le propyle parabène comme seule source de carbone (Close et Nielsen, 1976). Une souche de *Pseudomonas aeruginosa* capable de croître et de dégrader la formulation antimicrobienne contenant 0.02% de méthyle parabène et 0.01% de propyle parabène utilisé dans une formulation de gouttes ophthalmologiques a aussi été isolée (Hugo et Foster, 1964). Une autre souche de *P. aeruginosa*, isolée d'une formulation orale sans agents de préservation, s'est révélée capable de croître dans un milieu de sels minéraux contenant du propyle parabène ou un mélange de méthyle et propyle parabènes, et était capable d'hydrolyser les deux parabènes (Zedan et Serry, 1982; Zedan et Serry, 1984).

L'acide 4-hydroxybenzoïque produit par l'hydrolyse du lien ester des parabènes peut être utilisé à travers deux voies métaboliques distinctes. La première voie requiert l'oxygénation aérobique de l'acide 4-hydroxybenzoïque à la position *meta* pour former l'acide 3,4-dihydroxybenzoïque (acide protocatéchuique). Ce mécanisme a été documenté dans une souche de *P. aeruginosa* résistante aux parabènes et isolée d'une formulation orale sans agents de préservation (Zedan et Serry, 1984). Ce mécanisme a aussi été retrouvé chez plusieurs autres espèces bactériennes capables de métaboliser l'acide 4-hydoxybenzoïque mais non nécessairement résistantes aux parabènes, incluant des espèces de *Pseudomonas* (Harwood et Parales, 1996; Juhani et Kurjenluoma, 1975). Le deuxième mécanisme de dégradation de l'acide 4-hydroxybenzoïque requiert une décarboxylation non-oxidative pour former le phénol, qui n'est pas métabolisé. Ce

mécanisme a été documenté chez quelques espèces bactériennes, incluant *P. aeruginosa* et d'autres pseudomonades, aussi bien que chez *Clostridium hydroxybenzoicum, Clostridium thermoaceticum* et *Klebsiella* (maintenant *Enterobacter*) *aerogenes* (Grant et Patel, 1969; Hsu *et al.*, 1990; Ornston et Stanier, 1966; Patel et Grant, 1969; Zhang et Wiegel, 1994).

Les espèces d'*Enterobacter* sont des micro-organismes ubiquitaires dans les sols et les eaux. Ces bactéries sont aussi retrouvées dans d'autres sources environnementales telles que les matières végétales et les produits laitiers. Les espèces d'*Enterobacter* telles que *E. cloacae* et *E. aerogenes* sont des micro-organismes commensaux existant dans le tractus gastro-intestinal (Gaston, 1988). Des études d'hybridation ADN-ADN ont démontré que *E. cloacae* est de 40 à 45% relié à *E. aerogenes*, et que *Enterobacter gergoviae* est de 28 à 40% relié à *E. cloacae* ou *E. aerogenes* (Ewing, 1986; Steigerwalt, 1976). Les diverses espèces d'*Enterobacter* ne sont pas des pathogènes humains primaires, mais sont capables de causer des infections opportunistes chez des patients hospitalisés ou affaiblis. De plus, l'utilisation fréquente d'antibiotiques béta-lactames a créé une pression sélective envers des souches d'*Enterobacter* plus résistantes, qui sont devenues une source importante d'infections nosocomiales (Gaston, 1988). Les deux espèces d'*Enterobacter* les plus fréquemment isolées dans un milieu clinique sont *E. cloacae* et *E. aerogenes*, ce dernier étant récemment devenu un pathogène hospitalier important (Arpin *et al.*, 1996; Gaston, 1988). Dans certains milieux, *E. aerogenes* a remplacé *Klebsiella pneumoniae* comme la troisième cause d'infections nosocomiales à Gram-négatif, après *E. coli* et *P. aeruginosa* (Davin-Regli *et al.*, 1996; Schaberg *et al.*, 1991).

Certaines infections nosocomiales spécifiquement dues à *Enterobacter* sont mentionnées dans la littérature. Par exemple, une épidémie dans une unité médicale de soins intensifs a été attribuée à trois souches multi-résistantes d'*E. aerogenes* présentes dans l'environnement, incluant sur les meubles et les surfaces des planchers. L'épidémie causée par ces souches d'*E. aerogenes* s'est répandue à travers l'hôpital à cause du transfert de patients de l'unité de soins intensifs à d'autres unités pendant leur

hospitalisation (Arpin *et al.*, 1996). D'autres cas sérieux d'infection par *Enterobacter*, telles que la bactériémie, ont été reliés à divers équipements hospitaliers, tels qu'une solution de dextrose 5% contaminée ou des formulations alimentaires orales, ainsi que des équipements spécialisés tels des bassins d'hydrothérapie dans des unités de brûlures ou des laryngoscopes dans des unités de soins intensifs (Gaston, 1988; Jukka *et al.*, 2001; Maki *et al.*, 1976). Un autre exemple d'épidémie nosocomiale dans une unité de soins intensifs a été reliée à des bouteilles de lotion pour mains fréquemment utilisées par l'unité. Des isolats de *P. aeruginosa* et de *Enterobacter agglomerans* ont été isolés d'une des six bouteilles testées. Des tests supplémentaires par le manufacturier sur des bouteilles non-ouvertes ont révélé la présence de *P. aeruginosa* et de *E. agglomerans*. L'identité des agents de préservation dans cette lotion n'a pas été specifiée, mais le manufacturier a conclu qu'il existait une décomposition de l'agent de conservation avec le temps, ce qui a permis la croissance bactérienne (Becks et Lorenzoni, 1995).

Une souche d'*E. cloacae* a été isolée d'un supplément alimentaire commercial stabilisé avec un mélange de méthyle et de propyle parabène. Il avait été conclu que cette souche devait contenir une enzyme qui lui permet de dégrader les parabènes. Le premier objectif des travaux sur cette souche d'*E. cloacae* est de confirmer un mécanisme de résistance aux parabènes par dégradation enzymatique. Cet objectif visait l'identification des produits de dégradation des parabènes et la description de leur voie de dégradation complète par la bactérie. Le deuxième objectif était de cloner et de séquencer le gène responsable de la dégradation des parabènes. La séquence du gène permettra de confirmer la classe de l'enzyme et d'établir des homologies à d'autres enzymes. D'autres souches de bactéries pouvant causer des infections nosocomiales, telles que *E. gergoviae*, *E. aerogenes*, *E. agglomerans*, *E. coli*, *Burkholderia cepacia* et *P. aeruginosa* seront aussi testées pour leur capacité d'hydrolyser les parabènes et pour la présence d'un gène homologue. Le troisième objectif consistait en la caractérisation des paramètres cinétiques de l'enzyme purifiée envers les parabènes et des substrats structurellement analogues et en présence d'inhibiteurs irréversibles se liant de manière covalente à des acides aminés spécifiques. L'enzyme modifiée par ces inhibiteurs et ses fragments

tryptiques seront caractérisés par spectrométrie de masse afin d'identifier des acides aminés possédant possiblement un rôle catalytique.

Chapitre 1

Une souche bactérienne contaminante a été isolée d'un supplément alimentaire commercial normalement bien stabilisé avec un mélange de méthyle et de propyle parabène à des concentrations de 1700 ppm (11.2 mM) et de 180 ppm (1.0 mM), respectivement. Cette souche bactérienne a été identifiée comme *E. cloacae* par son profil de réactions biochimiques API ("Analytical Profile Index", bioMérieux, Hazelwood, MO) et par la séquence de son rRNA 16S, et a été nommée souche EM. La souche EM était capable de croître dans un milieu liquide avec des concentrations de parabènes comparables à celles du supplément alimentaire, tandis que la croissance d'une souche de référence *E. cloacae* LSPQ 3022 était complétement supprimée à ces concentrations. La souche EM était aussi capable de croître dans un milieu liquide contenant de très hautes concentrations de méthyle parabène (4000 ppm, 26.3 mM). La souche EM a démontré la capacité de dégrader les parabènes en créant une zone d'éclaircissement autour des colonies sur milieu solide contenant de 0.1% à 0.5% de parabènes cristallisés, tandis qu'une zone d'éclaircissement n'a pas été observée avec la souche LSPQ 3022. Le produit de dégradation des parabènes par la souche EM a été identifié comme étant l'acide 4-hydroxybenzoïque. Une culture liquide de la souche EM a été capable d'hydrolyser 1000 ppm (6.6 mM) de méthyle parabène en deux heures, tandis que la même transformation a été accomplie en 15 minutes par un surnageant de cellules soniquées. De plus, la souche EM a été capable de convertir en phénol en moins de 5 heures l'acide 4-hydroxybenzoïque produit à partir de 500 ppm (3 mM) de méthyle, d'éthyle ou de propyle parabène. Le phénol produit s'est accumulé dans le milieu de culture et a réduit la croissance de la souche EM sans la supprimer. La transformation de l'acide 4-hydroxybenzoïque en phénol est une voie rarement observée sous des conditions aérobies, puisque la voie catabolique la plus fréquemment documentée est la voie du béta-cétoadipate caractérisée par la formation de l'acide protocatéchuique.

HYDROLYSIS OF ESTERS OF 4-HYDROXYBENZOIC ACID (PARABENS) AND THEIR AEROBIC TRANSFORMATION INTO PHENOL BY THE RESISTANT *ENTEROBACTER CLOACAE* STRAIN EM

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ABSTRACT

Enterobacter cloacae strain EM was isolated from a commercial dietary mineral supplement stabilized by a mixture of methyl- and propylparaben. It harbored a high molecular weight plasmid and was resistant to high concentrations of parabens. Strain EM was able to grow in liquid media containing similar amounts of parabens as found in the mineral supplement (1700 and 180 mg L^{-1} of methyl and propylparaben, respectively, or 11.2 and 1.0 mM) and in very high concentrations (3000 mg L^{-1} or 19.7 mM) of methylparaben. This strain was able to hydrolyze approximately 500 mg L^{-1} (3 mM) of methyl-, ethyl- or propylparaben in less than two hours in liquid culture and the supernatant of a sonicated culture, after a 30-fold dilution, was able to hydrolyze 1000 mg L^{-1} (6.6 mM) of methylparaben in 15 minutes. The first step of paraben degradation was the hydrolysis of the ester bond to produce 4-hydroxybenzoic acid, followed by a decarboxylation step to produce phenol under aerobic conditions. The transformation of 4-hydroxybenzoic acid into phenol was stoichiometric. The conversion of approximately 500 mg L^{-1} (3 mM) of parabens to phenol in liquid culture was completed within five hours without significant hindrance to the growth of strain EM, while higher concentrations of parabens partially inhibited its growth.

INTRODUCTION

The esters of 4-hydroxybenzoic acid, also called parabens, are widely used as antimicrobial agents in a large variety of food, pharmaceutical and cosmetic products (20) due to their excellent antimicrobial activities and low toxicity (11). They are stable, effective over a wide pH range, and active against a broad spectrum of microorganisms. However, their mode of action is not well understood. They are postulated to act by disrupting membrane transport processes (7), by inhibiting DNA and RNA synthesis (17) or some key enzymes, such as ATPases and phosphotransferases in some bacterial species (15). Propylparaben is considered more active against most bacteria than methylparaben. However, because the latter has a greater solubility in water, they are often used as a mixture in commercial preparations.

Batches of a dietary mineral supplement normally well stabilized with a mixture of methyl- and propylparaben have shown signs of microbial contamination in conjunction with the disappearance of the parabens. Very little has been established with regards to microbial resistance and degradation pathways with respect to parabens. There are few cases in the scientific literature of microbial growth in paraben-stabilized products, although resistance to parabens by strains of *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Cladosporium resinae* has been reported (4, 27, 29, 31). However, as parabens are prominent antimicrobial agents in a variety of industries, the economic and medical impact of their degradation by microbial contaminants can be significant. Here, we report the characterization of a highly-resistant strain of the ubiquitous bacterium *Enterobacter cloacae* isolated from a paraben-stabilized mineral supplement and the identity of the main degradation products generated by this strain.

MATERIALS AND METHODS

Materials: Chemicals were obtained as follows: 4-hydroxybenzoic acid esters, 4-hydroxybenzoic acid, protocatechuic acid and bovine albumin from Sigma-Aldrich (St. Louis, MO), phenol from Fluka (Buchs, Switzerland), bis-trimethylsilyltrifluoroacetamide (BSTFA) from Pierce (Rockford, IL), ethyl acetate and acetonitrile from EM Science (Gibbstown, NJ), acetic acid from Mallinckrodt (Pointe-Claire, Qc., Can.), ultra-pure sucrose from GibcoBRL (Life Technologies, Inc., Gaithersburg, MD), Tryptic Soy Broth, Tryptic Soy Agar and all other growth media from Difco Laboratories (Detroit, MI) and all other chemicals from Anachemia (Ville Saint-Pierre, Qc., Can.). Antibiotics were purchased as follows: streptomycin, penicillin, trimethoprim, polymyxin B and norfloxacin, from Sigma (St-Louis, MO); rifampicin and chloramphenicol from Boehringer-Mannheim (Mannheim, Germany); tetracycline, erythromycin, ampicillin, and gentamicin from ICN Biomedicals (Aurora, OH) and kanamycin from Fisher (Fair Lawn, NJ). Restriction endonucleases were purchased from Pharmacia Biotech (Baie d'Urfé, Qc., Can.) and molecular weight markers from MBI Fermentas (Vilnius, Lithuania).

Growth conditions: Solid media containing paraben crystals was prepared by autoclaving Tryptic Soy Agar, and while still hot, 5 g L^{-1} of methylparaben or 1 g L^{-1} of propylparaben were added. Immediately after pouring, the Petri dishes were cooled at 4°C, which caused the parabens to form small, but clearly visible, crystals within the agar (4). The plates were incubated at 30°C overnight and the disappearance of the crystals around growing bacteria was monitored as an indication of paraben degradation. Liquid cultures of Tryptic Soy Broth with parabens were prepared by autoclaving media already containing parabens. Paraben stability during autoclaving was verified by HPLC. Determination of the optimum growth temperature was performed in Tryptic Soy Broth with and without a mixture of methyl- and propylparaben at concentrations similar to those of the mineral supplement. Cell growth in liquid media were monitored by optical

density readings at 600 nm, which were always measured below an OD of 0.3 after dilution, and where a reading of 1.0 OD corresponded to 6 x 10^8 cells/mL. All cultures for subsequent assays were grown at 30°C using a rotary agitator at 250 rpm under aerobic conditions.

Plasmid extraction : *E. cloacae* strains EM and E were cultured in Tryptic Soy Broth in the absence of parabens. The alkaline lysis protocol for plasmid extraction was followed as described by Sambrook *et al.* (25) and plasmid DNA was purified by CsCl gradient centrifugation (25). Plasmid transfer experiments were attempted by transformation of competent cells of strain E treated with calcium chloride according to the method of Sambrook *et al.* (25), by transformation of commercially-prepared competent cells of *E. coli* XL1-Blue (Stratagene, LaJolla, CA) as well as by electroporation of strain E according to the method of Smith and Iglewski (26) using the Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, CA).

Paraben sensitivity: Minimum inhibitory concentrations (MICs) were determined in Tryptic Soy Broth according to the method of Eklund (6) with the following modifications: a small aliquot of a culture grown overnight in Tryptic Soy Broth was diluted into Tryptic Soy Broth medium having been autoclaved with the appropriate concentrations of parabens such that the starting optical density of the culture was approximately 0.05 at 600 nm. The density of the bacterial suspensions was measured immediately after dilution, after 24 and 48 hours of incubation at 30°C with shaking. The minimum inhibitory concentrations were defined as the amounts of preservative added to the media that prevented an increase in the optical density of the cell suspension after 48 hours relative to the density immediately after inoculation.

Characterization of hydrolytic activity: Liquid cultures were prepared from EM cells grown in Tryptic Soy Broth overnight, centrifuged and resuspended in fresh medium before being inoculated into Tryptic Soy Broth containing the appropriate paraben. The

optical density of all cultures was monitored in the same manner as for the MICs. The cells were incubated at 30°C with shaking and at timed intervals, 1 mL aliquots were removed for HPLC analysis and heated immediately to 80°C for 10 minutes in order to prevent further enzymatic degradation of the parabens. Cell lysates of strain EM grown in Tryptic Soy Broth without parabens were prepared with an ultrasonicator probe (Heat Systems, Inc., Farmingdale, NY) using three 20-second pulses at 143 W, followed by centrifugation at 16000*g* for 15 minutes. A 30-fold dilution of the cell lysate was subsequently made in Tryptic Soy Broth containing 1000 mg L^{-1} (6.6 mM) of methylparaben and incubated for 2 hours at 30°C with shaking. Sampling was performed at timed intervals as described above and a control was prepared in the same manner from an EM culture that had not been sonicated. Total protein concentration was measured in the cell-free extracts of sonicated and non-sonicated cultures with the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) according to the method of Bradford (3), using bovine albumin as a standard.

Identification of paraben degradation products: The metabolites of the parabens were identified both by HPLC and GC/MS. The HPLC analyses were performed on a HP 1100 (Agilent Technologies, Kirkland, Qc.) equiped with a 150 mm x 4 mm C_{18} reverse-phase Hypersil ODS column (5 µm) (Agilent Technologies) and a variable-wavelength UV detector. A water/acetonitrile gradient containing a constant concentration of 0.1% acetic acid was used, starting with 90% water and ending with 90% acetonitrile after five minutes at a flow rate of two mL/min. The GC was a Varian 3500 (Varian Canada, St-Laurent, Qc.) equipped with a DB-5 column (30 m, 320 µm i.d., film thickness 0.25 µm). The carrier gas was helium at a flow rate of 2.2 mL/min. The temperature gradient started at 70°C, rose to 250°C at 10°C/min, and to 310°C at 25°C/min. The mass spectrometer was a Finnigan Ion Trap 800 (Thermo Quest, Schaumburg, IL) and the scan range was from 70 to 440 Da.

The samples were prepared for HPLC analysis by centrifugation of the cell suspensions to remove cell debris and adding acetonitrile containing 1% acetic acid to the supernatants to a final concentration of 10%. The parabens, 4-hydroxybenzoic acid and phenol were identified according to their retention times and quantified with the appropriate calibration curves. Sample preparation for GC/MS analysis was performed by homogenizing the cell cultures by vortexing, saturating with sodium chloride and extracting with ethyl acetate. The organic layer was dried with anhydrous sodium sulfate and the compounds retained in the organic layer were derivatized by direct addition of bis-trimethylsilyltrifluoroacetamide (BSTFA) and heating at 70°C before injection. The identification of the trimethylsilyl derivatives of 4-hydroxybenzoic acid and phenol was made by comparison of their retention times and mass spectra to those of standards derivatized in the same manner.

RESULTS AND DISCUSSION

Characterization of bacterial strains: The paraben-resistant *E. cloacae* strain EM was isolated from batches of a dietary mineral supplement which was normally well stabilized with methyl- and propylparaben. This supplement contained various mineral glycerophosphates, plant extracts, and methyl- and propylparaben at 1.7 and 0.18 g L^{-1}, respectively. The contamination was observed simultaneously in batches manufactured at distant production plants, indicating that the origin of the contaminants was not environmental but that they were probably introduced from one of the ingredients of the mixture. The most obvious sign of bacterial growth was the inflation of the plastic bottles due to the production of gas. A facultative anaerobe, Gram-negative short rod was isolated from these bottles and identified as *E. cloacae* by API 20E galleries (bioMerieux, St-Laurent, Qc., Can.) and named strain EM. The identification was further verified by comparing it to a collection strain *E. cloacae* LSPQ 3022 (Laboratoire de Santé Publique du Québec, St-Anne de Bellevue, Qc., Can.), named strain E. Strain EM formed large smooth colourless colonies with an aspect and colour identical to those of the reference strain E on Tryptic Soy Agar, blood, MacConkey and Hektoën media. The identification was confirmed by sequencing a 591 bp PCR-amplified portion of the 16S rRNA gene using the universal eubacterial primers 5'AGAGTTTGATCCTGGCTCAG3' (nucleotides 8-27) and 5'AAGGAGGTGATCCAGCCGCA3' (nucleotides 1522-1541) for *E. coli* (GeneBank accession no. J01695). A similarity >99% was obtained compared to the sequence of the *E. cloacae* 16S rRNA gene (ATCC 13047T, GeneBank accession no. AJ251469). Strain EM grew in Tryptic Soy Broth with the production of gas at temperatures between 25 and 37°C and its optimal growth temperature was found to be 30°C with or without parabens (data not shown). Comparative antibiograms of the two strains demonstrated that, as previously reported for this species, both *E. cloacae* strains showed a resistance to high concentrations (100 µg/mL) of penicillin and ampicillin (21, 30) and were equally resistant to 100 µg/mL of erythromycin and to 50 µg/mL of trimethoprim. Both strains were found to be sensitive to 25 µg/mL of streptomycin, as

well as to 10 μg/mL of tetracycline or polymyxin B and to 0.5 μg mL of norfloxacin. Strain EM was found to differ from the reference strain E by being sensitive to 50 μg/mL of rifampicin and to 10 μg/mL of either chloramphenicol, gentamicin and kanamycin, although at 50 μg/mL of the three latter compounds, both strains became sensitive.

Plasmid characterization. Resistance to biocides is often associated with plasmids (24). A large plasmid was detected in strain EM while no plasmid was found in the reference strain E. The plasmid in strain EM was extracted and purified by CsCl gradient centrifugation and its molecular weight was estimated at 100 = 20 kb. High molecular weight plasmids have previously been found in *E. cloacae* isolates from nosocomial infections that had a high level of multi-resistance (14, 22). Transfer of the plasmid from strain EM by transformation of strains *E. cloacae* E and of *E. coli* XL1-Blue did not yield transformants able to hydrolyze crystallized parabens on Tryptic Soy Agar. Further attempts to transfer the plasmid to strain E by electroporation did not yield transformants able to grow on plates containing 2500 mg L^{-1} (15.0 mM) of crystallized ethylparaben, suggesting that the large size of the plasmid may not permit it to cross the bacterial membrane. Strain EM was also not cured of its plasmid by growing cultures at 42°C or in ethidium bromide. Interestingly, strain EM was able to grow in the presence of concentrations of ethidium bromide as high as 1000 mg L^{-1} (2.5 mM). Such resistance has been associated with plasmid-mediated efflux in *Staph. aureus* and has been cloned into *E. coli* (23).

Resistance to parabens. Both strains E and EM grew to very high cell densities in Tryptic Soy Broth alone (Fig. 1A), with the reference strain E reaching slightly higher densities than strain EM. However, strain EM grew in the presence of methyl- and propylparaben at similar concentrations as those present in the mineral supplement (1400 mg L^{-1} and 150 mg L^{-1}, respectively, or 9.2 and 0.83 mM), while at these concentrations, the growth of strain E was suppressed (Fig. 1A). Due to limitations in the solubility of propylparaben, the effect of similar concentrations of the series methyl-, ethyl- and

propylparaben could only be studied at approximately 500 mg L^{-1} (3 mM). The growth of the reference strain E was considerably hindered by concentrations of 400 and 500 mg L^{-1} of methyl- and ethylparaben, respectively, as it reached ODs of only 4.5 and 2.4, after 24 hours (Fig. 1B), and was suppressed by 400 mg L^{-1} of propylparaben. The optical density of strain EM at these paraben concentrations was comparable to the growth obtained in Tryptic Soy Broth alone, reaching an OD of 3 after five hours and of 9 after 24 hours for all three parabens (results not shown). Furthermore, strain EM survived in very high concentrations of methylparaben (3000 mg L^{-1} or 19.7 mM) after 24 hours and was further able to grow, while the growth of strain E was suppressed at concentrations of 2000 mg L^{-1} (13.1 mM) and remained significantly below that of strain EM at lower paraben concentrations (Fig. 1C).

The differences in the antimicrobial activity of methyl-, ethyl- and propylparaben are clearly demonstrated by the effects of similar concentrations on the growth of the paraben-sensitive strain E (Fig. 1B). Propylparaben was the most effective of the three, as 400 mg L^{-1} (2.2 mM) suppressed the growth of strain E, while similar amounts of ethyl- or methylparaben did not inhibit cell growth entirely. The stronger antibacterial action of propylparaben may be due to its greater solubility in the bacterial membrane, which may allow it to reach cytoplasmic targets in greater concentrations. However, since a majority of the studies on the mechanism of action of parabens suggest that their antibacterial action is linked to the membrane (6, 7), it is possible that its greater lipid solubility disprupts the lipid bilayer, thereby interfering with membrane transport processes and perhaps causing the leakage of intracellular constituents.

Paraben resistance of strain EM relative to strain E is also reflected in the minimum inhibitory concentrations (MICs) of the four most commonly used parabens. The methylparaben concentration required to suppress the growth of EM was 4000 mg L^{-1} (26.3 mM). Due to limitations in their solubilities, the concentrations of ethyl-, propyl- and butylparaben required to prevent growth of strain EM could only be

determined as higher than 1600, 600 and 200 mg L^{-1} (9.6, 3.3 and 1.0 mM), respectively, as this strain was able to reach very high optical densities (OD > 7 at 600 nm) at these concentrations. In comparison, the MICs for strain E of methyl-, ethyl- and propyl paraben were 2000, 800 and 600 mg L^{-1} (13.1, 4.8 and 3.3 mM), respectively, while butyl paraben limited the growth of strain E considerably without completely suppressing it at 200 mg L^{-1} (1.0 mM). The MICs reported in the literature for these four parabens towards *E. cloacae* ATCC 23355 are 1000, 1000, 500 and 250 mg L^{-1} (6.6, 6.0, 2.8 and 1.3 mM) (11), respectively, showing the remarkable resistance of strain EM towards these compounds. Due to the high resistance levels of strain EM to all four parabens, as evidenced by the MICs, the minimum bactericidal concentrations (MBCs) were not determined.

Degradation of parabens by strain EM. When a 10 µL aliquot of an overnight culture of EM grown in Tryptic Soy Broth without parabens was deposited in the middle of a Tryptic Soy Agar plate containing 1, 5 or 10 g L^{-1} of crystallized propylparaben, a time-dependent clearance zone around the growing bacteria was observed within a few hours. After eight days of incubation, the diameter of this zone on 1 g L^{-1} of propylparaben had increased to the extent that the paraben crystals over the entire area of a standard 100 mm Petri dish had disappeared, although the diameter of the bacterial spot where the cells were initially deposited did not increase significantly (results not shown). Furthermore, when a small area of agar cleared of crystallized parabens by strain EM, but outside the diameter of bacterial growth, was transferred to liquid media containing 1000 mg L^{-1} of methylparaben (6.6 mM), only 7 % of the original amount of paraben remained after 24 hrs and 0.02 % of the paraben was found in the media after 48 hrs. No evidence of cell growth was detected at these time points, demonstrating that the factor responsible for paraben degradation diffused through agar outward from the perimeter of bacterial growth (results not shown). Additionally, when a culture filtrate (0.2 µm) of strain EM was placed on Tryptic Soy Agar containing crystallized propylparaben, a 2.5 cm clearance zone was observed after a 24 hr incubation. No clearance zone was observed

with a EM filtrate heated to boiling, indicating that the factor was heat-sensitive and probably enzymatic in nature. Additionally, no clearance zone was observed with a filtrate of strain E (results not shown). Hence, the considerable growth of strain EM in methyl-, ethyl- and propylparaben above their reported MICs (11) and in comparison with the control strain E can be explained in terms of the enzymatic degradation of these antibacterial agents.

Conversion of parabens to 4-hydroxybenzoic acid. When strain EM was incubated at 30°C overnight in Tryptic Soy Broth containing similar concentrations of methyl- and propylparaben as in the mineral supplement (1400 and 150 mg L^{-1}, respectively, or 9.2 and 0.83 mM), complete disappearance of both parabens was observed within 24 hrs of incubation at 30°C, while no significant change occurred in the concentrations of parabens present in a parallel culture of strain E (results not shown). Additionally, when the supernatant of a culture of strain EM grown without parabens was diluted 1:30 and inoculated into Tryptic Soy Broth containing 1000 mg L^{-1} (6.6 mM) of methylparaben. only 6% (0.4 mM) of the initial paraben remained in the medium after two hours (Fig. 2). The disappearance of the paraben was accompanied by a corresponding increase in the amounts of 4-hydroxybenzoic acid, which reached 900 mg L^{-1} (6.5 mM) after two hours. In order to assess if the enzyme responsible for paraben hydrolysis was extra- or intracellular, the same EM culture was sonicated and the supernatant was equally diluted and placed in 1000 mg L^{-1} (6.6 mM) of methylparaben (Fig. 2). It was found that only 0.06% (0.004 mM) of methylparaben remained in the media after 15 minutes of incubation, while 80% of the paraben still remained in the supernatant of the non-sonicated culture after the same time, resulting in a hydrolysis rate difference greater than 800-fold, while the amount of protein released after sonication was only 69-fold greater (1.1 mg/mL). This extremely rapid hydrolysis by the sonicated cell suspension was paralleled by the rapid appearance of 950 mg L^{-1} (6.8 mM) of 4-hydroxybenzoic acid. The nature of the degradation product and the differences between its rate of formation

by the sonicated and the non-sonicated cultures indicate that the enzyme may be of intracellular nature or that it may be targeted to the periplasm.

Transformation of 4-hydroxybenzoic acid into phenol. Strain EM grown without parabens was inoculated into Tryptic Soy Broth containing 400 mg L^{-1} (2.6 mM) of methylparaben and after 60 minutes, transformed more than 99% of the paraben into 4-hydroxybenzoic acid (Fig. 3). However, the acid produced accumulated in the medium only transiently, as more than 99.9% was transformed into phenol after five hours of incubation (Fig. 3). The kinetics of paraben hydrolysis and phenol formation were nearly identical for ethyl- and propylparaben at similar concentrations (results not shown). No cell lysis, indicated by decreases in optical density, was observed in cultures of EM in medium containing approximately 500 mg L^{-1} (3 mM) of either of the three parabens after five hours of incubation, at which point phenol reached its maximal concentration (Fig. 3). Further confirmation of the origin of phenol was obtained from the 1:1 stoichiometric conversion of 4-hydroxybenzoic acid to phenol obtained by incubating strain EM with 700, 1500, 2300, 2400 and 3000 mg L^{-1} (4.6, 9.9, 15.1, 15.8 and 19.7 mM) of methylparaben. A linear relationship with a slope of 1.0 was established between the amount of 4-hydroxybenzoic acid produced from the parabens and the amount of phenol present after the complete disappearance of the acid (results not shown). It is reported in the literature that 800 mg L^{-1} (8.5 mM) of phenol induced a lag phase in *E. aerogenes*, a species closely related to *E. cloacae* (28), during which the cells nonetheless remained viable (5). This concentration is close to the 900 mg L^{-1} (9.6 mM) of phenol which accumulated in the media when strain EM was grown in a mixture of 1400 mg L^{-1} (9.2 mM) of methylparaben and 150 mg L^{-1} (0.83 mM) of propylparaben. The growth of strain EM at these concentrations of parabens was considerably reduced after 24 hours in comparison with EM grown in Tryptic Soy Broth alone (Fig. 1A). The main difference with this latter experiment, aside from the presence of rapidly degraded parabens, is the transient accumulation of 4-hydroxybenzoic acid and subsequently of phenol (results not

shown). This suggests that these compounds may be responsible for the reduced growth over 24 hours of strain EM initially cultivated in the presence of parabens.

The decarboxylation of 4-hydroxybenzoic acid into phenol by aerobic bacteria has been reported only once with a strain of *Enterobacter aerogenes*, a bacterium closely related to *E. cloacae* (28), both under aerobic (19) and anaerobic (10) conditions. The usual degradation pathway of 4-hydroxybenzoic acid by aerobic bacteria is through the β-ketoadipate pathway, resulting in the formation of protocatechuic acid instead of phenol (Fig. 4). This pathway and the enzymes necessary for the degradation reactions, coded by the *pca* operon, are highly conserved and have been extensively characterized in several prokaryotes, including *Acinetobacter calcoaceticus*, *Pseudomonas putida* and *Agrobacterium tumefaciens* (12, 18). The metabolism of the 4-hydroxybenzoic acid generated from parabens by a resistant *Pseudomonas aeruginosa* strain was also found to proceed through the formation of protocatechuic acid (31). In the present study, no protocatechuic acid was detected by HPLC or GC/MS during paraben degradation by strain EM. Instead, the decarboxylation of 4-hydroxybenzoic acid to phenol proceeded stoichiometrically (Fig. 4). It has been documented that under anaerobic conditions, 4-hydroxybenzoic acid can be decarboxylated into phenol. This pathway has been found in a number of anaerobic consortia isolated from the environment (1, 8, 33) as well as in *Clostridium hydroxybenzoicum* and *Moorella* (bas. *Clostridium) thermoacetica* (13, 32). However, the aerobic transformation of 4-hydroxybenzoic acid into phenol is a rarely-documented pathway and raises questions about the ability of other ubiquitous *Enterobacteriaceae* to carry out these reactions.

The utilization of parabens as growth substrates by various bacterial genera has been observed by Beveridge and Hart (2). Close and Nielsen (4) have reported hydrolysis of the parabens and their utilisation as sole carbon source, while Suemitsu *et al.* (29) reported the formation of 4-hydroxybenzoic acid as a degradation product, both by strains of *Pseudomonas* (*Burkholderia*) *cepacia*. However, in these studies, low concentrations

of parabens were used (less than 100 mg L^{-1}) and more than two to four weeks were required to achieve complete degradation. Similarly, a *P. aeruginosa* strain isolated by Zedan and Serry required five days to completely hydrolyze 100 mg L^{-1} of propylparaben, while the *Cladosporium* strain isolated by Sokolski *et al.* was capable of hydrolysing 70% of a 2000 mg L^{-1} paraben solution in five days (27, 31). In contrast, strain EM was capable of completely hydrolysing approximately 500 mg L^{-1} of methyl-, ethyl- or propylparaben in less than two hours, and a mixture of 1400 and 150 mg L^{-1} of methyl- and propylparaben, similar to amounts used in commercial preparations, in less than four hours, demonstrating the remarkable activity of this strain towards parabens. To our knowledge, this is the first report of a strain of *E. cloacae* that can inactivate high amounts of parabens in its early stages of growth and can continue to grow in high concentrations of 4-hydroxybenzoic acid and phenol. The introduction of *Enterobacter* species as clinical pathogens has previously been reported for strains of *E. cloacae* present in contaminated dextrose infusion fluid or from sources as varied as formulated oral feeds, hydrotherapy tanks or liner caps of intravenous fluid bottles (9, 16). The resistance of strain EM to such common preservatives as parabens can engender health-related concerns, as they are used in a number of pharmaceutical products, which might create the potential for the spread of paraben-resistant bacteria as nosocomial pathogens.

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FIGURE LEGENDS

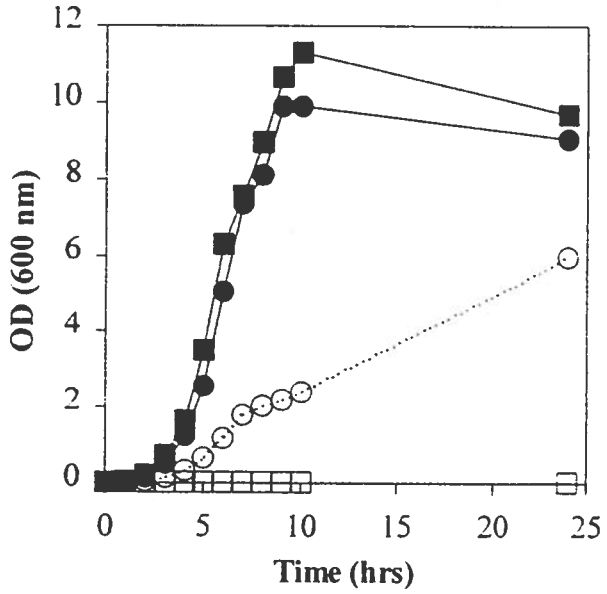
Figure 1: Growth of strains EM (●/○) and E (■/□) in A) Tryptic Soy Broth (—) and growth in Tryptic Soy Broth containing 1400 and 150 mg L^{-1} of methyl- and propylparaben (9.2 and 0.83 mM), respectively (·····); B) Growth of strain E in the presence of 400 mg L^{-1} of methylparaben (2.6 mM) (○) and of propylparaben (2.2 mM) (□) and 500 mg L^{-1} of ethylparaben (3.0 mM) (◊) and C) Growth of strains EM (○) and E (□) after 24 hours in Tryptic Soy Broth with increasingly high concentrations of methylparaben.

Figure 2: Conversion of 1000 mg L^{-1} (6.6 mM) of methylparaben (●/○) into 4-hydroxybenzoic acid (■/□) by the supernatants of sonicated (—) and non-sonicated (·····) cultures of strain EM, yielding 950 and 900 mg L^{-1} (6.9 and 6.5 mM) of 4-hydroxybenzoic acid, respectively. The appearance of the degradation product was monitored by HPLC.

Figure 3: Complete transformation of methylparaben by strain EM in Tryptic Soy Broth. The paraben (●) at a concentration of 400 mg L^{-1} (2.6 mM) is rapidly hydrolyzed into 4-hydroxybenzoic acid (■), which is stoichiometrically decarboxylated into phenol (Δ). The appearance of the degradation products was followed by HPLC.

Figure 4: Degradation pathway of esters of 4-hydroxybenzoic acid into phenol by strain EM in aerated liquid culture. The initial hydrolysis of methylparaben produces 4-hydroxybenzoic acid and methyl alcohol. Further degradation of 4-hydroxybenzoic acid does not follow the protocatechuate pathway. Instead, the 4-hydroxybenzoic acid produced is stoichiometrically converted into phenol by a decarboxylase-type enzyme operating under aerobic conditions.

A)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Time (hrs)
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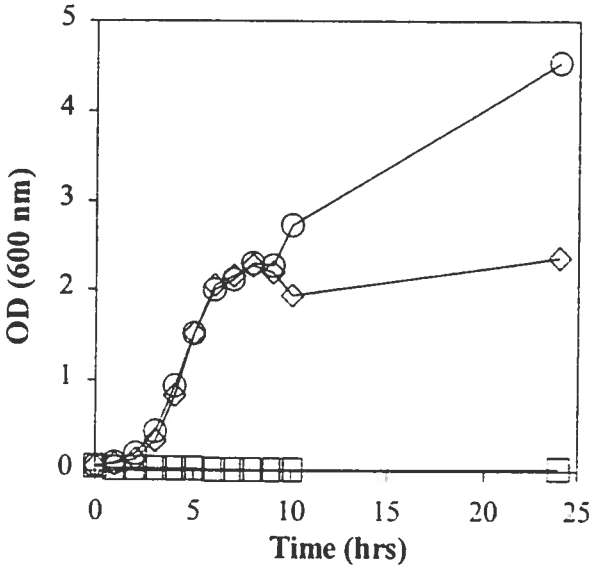
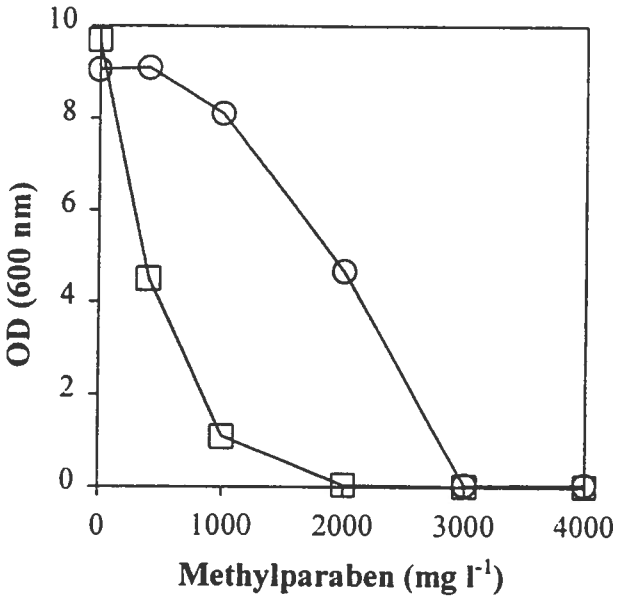
2
4
6
8
10
12
0
5
10
15
20
25

B)


OD (600 nm)
Time (hrs)
0
1
2
3
4
5
0
5
10
15
20
25

C)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Methylparaben (mg l-1)
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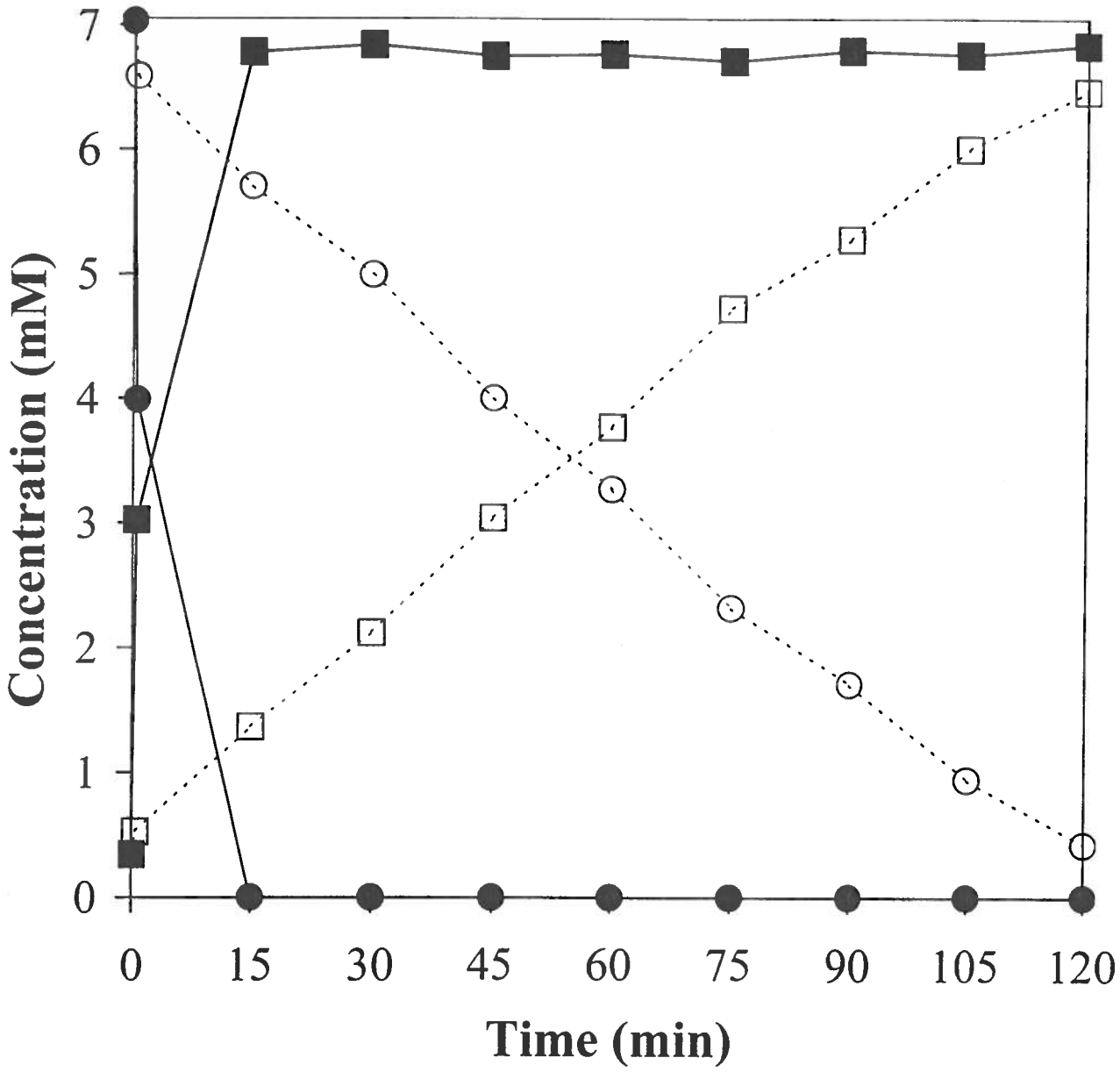
6
8
10
0
1000
2000
3000
4000


Concentration (mM)
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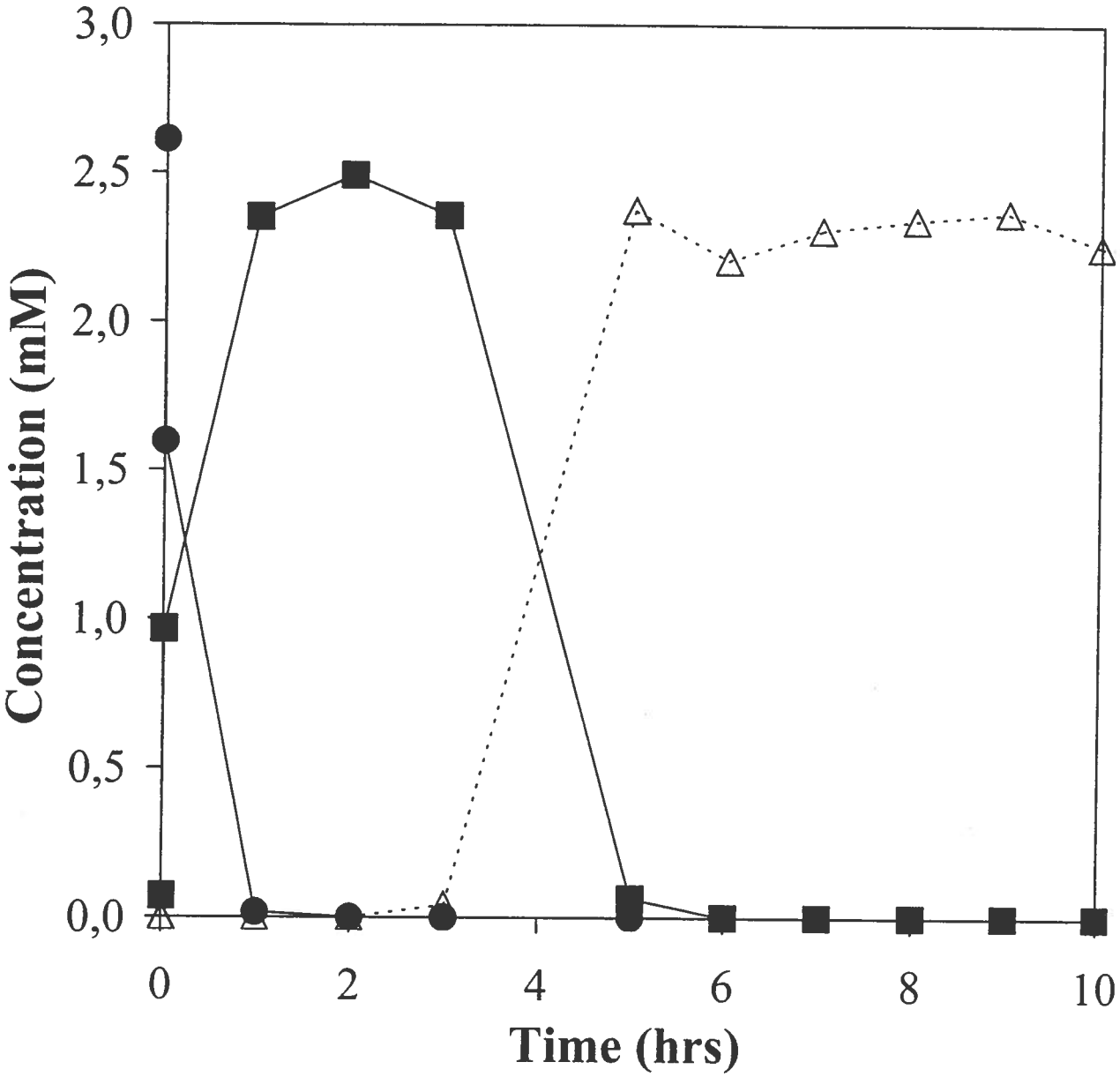
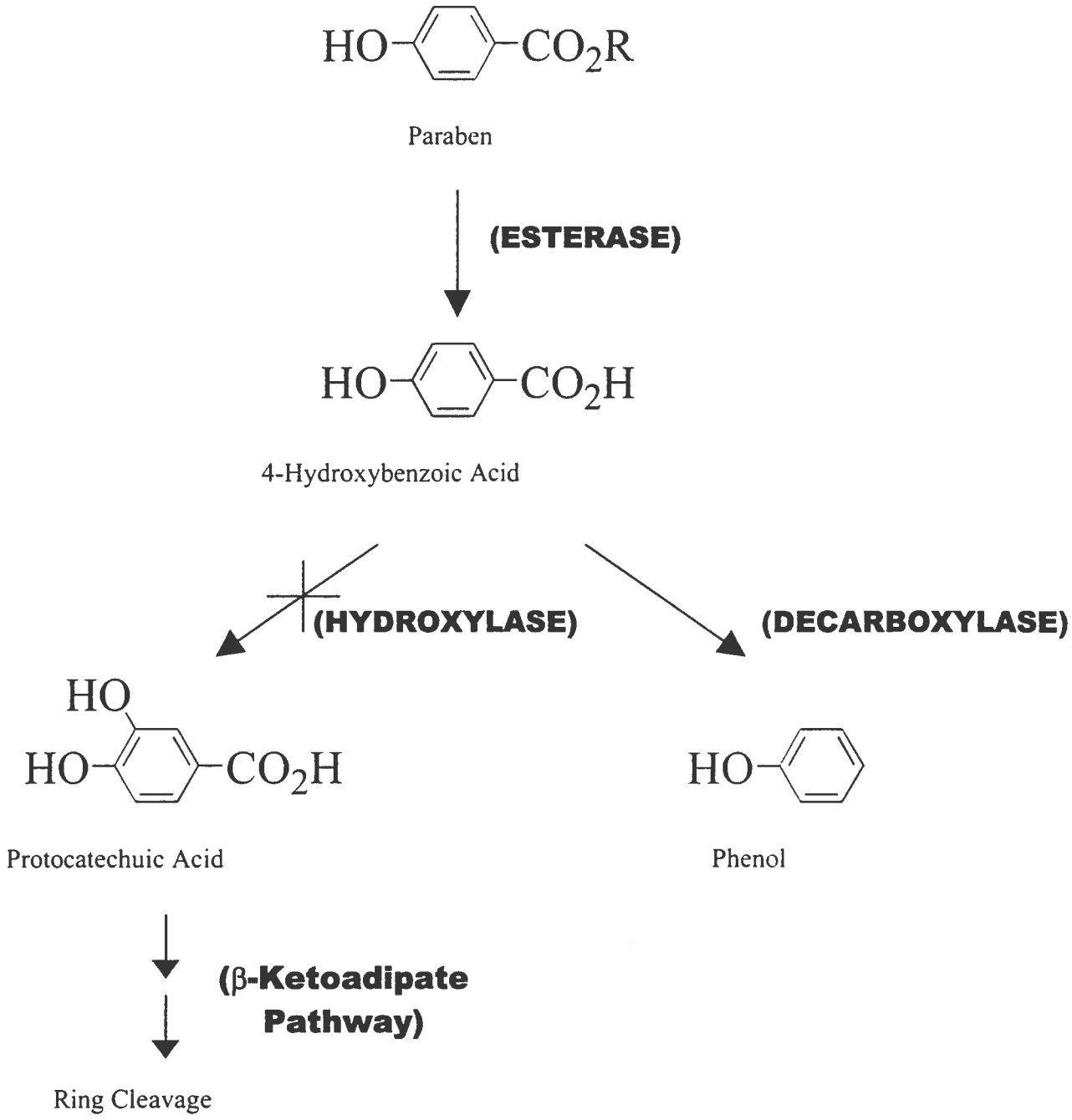
6
5
4
3
2
1
0
0
15
30
45
60
75
90
105
120
Time (min)


3,0
2,5
2,0
1,5
1,0
0,5
0,0
Concentration (mM)
0
2
4
6
8
10
Time (hrs)


HO
CO2R
Paraben
(ESTERASE)
HO
CO2H
4-Hydroxybenzoic Acid
(HYDROXYLASE)
(DECARBOXYLASE)
HO
HO
CO2H
HO
Protocatechuic Acid
Phenol
(β-Ketoadipate
Pathway)
Ring Cleavage

Chapitre 2

La souche d'*E. cloacae* EM contient un estérase capable de dégrader les esters de l'acide 4-hydroxybenzoïque (parabènes) en acide 4-hydroxybenzoïque. Le gène codant pour cette estérase, nommé *prbA*, a été cloné et séquencé. L'approche de clonage a d'abord amplifié une région de 300 pb du N-terminal en utilisant des amorces dégénérées. Cette région a été marquée avec de la digoxigénine et hybridée à l'ADN chromosomique de la souche EM pour identifier des fragments contenant le gène, qui ont été amplifiés par PCR Inverse. La séquence du gène *prbA* entier contenait 1602 nucléotides, codant pour 533 acides aminés. Les premiers 93 nucléotides (acides aminés 1 – 31) ont été identifiés comme un peptide signal, formant une protéine mature de 502 acides aminés d'un poids moléculaire théorique de 54.6 kDa. La séquence de *prbA* contenait un motif signature et un motif de site actif d'une carboxylestérase de type B. Plusieurs estérases eucaryotiques présentes dans les banques de données ont montré un haut facteur d'identité par rapport à *prbA*, incluant des protéines d'origine humaine, de rat et de souris. Un certain nombre d'estérases procaryotiques ont aussi demontré une haute homologie avec *prbA*, notamment celles de *Deinococcus radiodurans*, *Delftia acidovorans* et *Salmonella* sp. Le transfert du gène *prbA* à la souche sensible aux parabènes d'*E. coli* DH5α a resulté en l'acquisition d'une capacité hydrolytique envers les parabènes comparable à celle de la souche EM. Deux souches supplémentaires d'*E. gergoviae* et une souche de *Burkholderia cepacia* ont démontré la capacité de dégrader les parabènes en acide 4-hydroxybenzoïque et un gène dont la partie N-terminale est à plus de 95% identique à celle de *prbA* a été identifié chez les deux souches d'*E. gergoviae*. Ces souches ont récemment été isolées de produits commerciaux contaminés en France, suggérant la présence d'homologues proches de *prbA* dans des endroits géographiquement éloignés.

prbA, A GENE CODING FOR AN ESTERASE HYDROLYSING PARABENS IN *ENTEROBACTER CLOACAE* AND *ENTEROBACTER GERGOVIAE* STRAINS

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Running Title: Parabens hydrolysis by PrbA from *E. cloacae*

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ABSTRACT

The new gene *prbA* encodes an esterase responsible for the hydrolysis of the ester bond of parabens in the *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, of which the first 31 represent a proposed signal peptide, yielding a mature protein of a putative molecular weight 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 hydrolyse 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 towards methyl paraben. The presence of homologues of *prbA* was tested in additional ubiquitous bacteria which may be causative factors in opportunistic infections, including *E. gergoviae*, *E. 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 *B. cepacia* were able to degrade almost completely 800 mg l^{-1} of methyl paraben. Two strains of *E. gergoviae*, named G1 and G12, contained a gene which 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 which represent a common source of opportunistic infections.

INTRODUCTION

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 utilise 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 which 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 1700 mg L^{-1} (11.2 mM) of methyl paraben and 180 mg L^{-1} (1.0 mM) of propyl paraben. 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 from ICN Biomedicals (Aurora, OH). Methyl paraben was obtained from Sigma-Aldrich (St. Louis, MO) polyethylene glycol was from A & C American Chemicals (Fair Lawn, NJ) and agarose was from Life Technologies (Grand Island, NY). DNA was purified from agarose with the Qiaex II gel extraction kit from Qiagen (Mississauga, ON, 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, QC). Lysozyme was from Roche (Laval, QC, Canada) and proteinase K from Qiagen. All reagents for PCR reactions, including *Taq* polymerase, were from Pharmacia Biotech. All reagents for ligation, including T4 DNA ligase, were included in the pGEM-T Easy Vector System I from Promega (Madison, WI). Digoxigenin (DIG)-labeled DNA was constructed with the DIG DNA labeling kit from Roche according to the manufacturer's instructions. Nylon membranes were from Roche and Biomax MR Scientific Imaging Film was from Kodak (Rochester, NY).

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)GA(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 SDS-PAGE band, and consisting of the amino acids QELSPVVQMSKGTIEGVKND. As the N-terminus 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, of the sequence PVMVWIHG, were suitable for the construction of the second primer, EcoPep4, as 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 Touch-down 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 minutes and 94°C for 5 minutes (1 cycle); 60°C for 1 minute, 72°C for 2 minutes and 94°C for 40 seconds (3 cycles); repeats of the previous parameters at decreasing 2°C increments until 42°C for 3 cycles each; 40°C for 1 minute, 72°C for 2 minutes and 94°C for 40 seconds (14 cycles) and 40°C for 1 minute, 72°C for 10 minutes (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 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 digoxigenin (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 Blot 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 kb 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_1R and L_2F, 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 fragments. The sequence of the reverse primer L_1R was 5' GGTTCGCTTTTAACGTGCCC 3' and that of the forward primer L_2F was 5' CACCAAGCTCACTTTGGGTG 3'. The PCR conditions were as follows: 80°C for 2 minutes, 94°C for 5 minutes and 55°C for 5 minutes (1 cycle); 72°C for 2 minutes, 94°C for 40 seconds and 55°C for 1 minute (30 cycles) and 72°C for 10 minutes. The amplified 2.8- and 3.0-kb fragments were subsequently ligated into pGEM-

T Easy and the same procedure was used for sequencing as described above for 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 – 257 in GenBank accession number 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 – 1843). Due to potential primer hairpin formation, 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 L^{-1} (4.0 mM) of methyl paraben 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 minimum inhibitory concentrations (MICs) were determined by inoculating a culture pre-grown in TSB to stationary phase in TSB containing 200, 400, 800, 1600 or 3200 mg L^{-1} of methyl paraben to an optical density of 0.05 at 600 nm, where 1.0 OD corresponded to 6 x 10^8 cells/mL of *Enterobacter*, and incubating for 24 hours at 30°C with shaking. Optical density readings were taken at the moment of inoculation and after 24 hours and the minimal inhibitory concentrations were defined as the minimal amount of methyl paraben that prevented an increase in the optical density of the suspension. To determine the efficiency of the strains to degrade parabens, the cells of exponentially-growing cultures at an optical density of 2.0 were harvested by centrifugation and resuspended in TSB

medium containing 800 mg L^{-1} (5.3 mM) of methyl paraben for a two-hour incubation period at 30°C with shaking. At the beginning and end of the 2-hour incubation, aliquots were removed and heated immediately to 80°C for 10 minutes 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 analysed by HPLC as previously described (26). The presence of homologues of *prbA* in selected strains was done using Touch-down PCR using the primers and conditions specified above.

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 Touch-down 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 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 Blot 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 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 Figs. 2C and 2D was used to clone the 3.0 kb fragment following digestion with *Eco*RI (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-kb and 3.0-kb fragments were sequenced on both the forward and reverse strand.

The nucleotide sequence of the esterase gene of *E. cloacae* strain EM, named *prbA* (parabenA) has been annotated and deposited in the GenBank public database. The coding sequence is composed of 1602 nucleotides, including the stop codon, constituting an immature protein of 533 amino acids with a theoretical molecular weight of 57.9 kDa (Protein General Sequence Analysis, UK HGMP Resource Centre) (29). The first 31-amino acid stretch of the protein (nucleotides 248 – 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 1-11 (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 weight 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 – 88 (EDCLYLNVYTP) as well as a carboxylesterase type-B serine active site between amino acids 176 – 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 hydrolyse 49% of 600 mg L^{-1} (4.0 mM) of methyl paraben within 30 minutes of inoculation and 97% after 90 minutes. In comparison, strain *E. cloacae* EM hydrolysed 93% of methyl paraben after 30 minutes and 99.6% after 90 minutes (Fig. 3). The slightly slower rate of hydrolysis in *E. coli*

compared to *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 3200 ppm for *E. coli* DH5α cells without the *prbA* gene to >3200 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 hr incubation at 1600 ppm of methyl paraben, in comparison with the unmodified *E. coli* DH5α strain that only presented a small OD increase from 0.155 to 0.207 during the same period.

Resistance to parabens in related strains. The resistance towards parabens of other strains closely related to *E. cloacae* EM was measured with three other *E. cloacae*, twenty-one *E. gergoviae*, five *E. aerogenes* and four *P. agglomerans* strains obtained from various commercial or clinical sources or from bacterial collections (Table 1). Three *E. coli* and two *P. aeruginosa* strains were selected as ubiquitous bacteria which may represent a health risk, and finally a strain of *B. cepacia* previously known to hydrolyse 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 minimal inhibitory concentration (MIC) of methyl paraben (Table 1). Although the MIC does not provide indications as to 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 the strains (except *P. agglomerans* LSPQ 3825) showed a high tolerance to this agent, with MICs of 1600 mg L^{-1} (10.5 mM) of methyl paraben or greater. In fact, the majority of the strains, of either commercial or clinical origin, showed a MIC of 3200 mg L^{-1}, regardless of the lack of previous known exposure to this biocide. The *E. cloacae* strain EM, containing the *prbA* gene, predictably showed a very high MIC, greater than 3200 mg L^{-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 L^{-1} (7, 16).

Interestingly, a strain of *E. gergoviae* (G1) also showed a MIC >3200 mg L^{-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 L^{-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, varying 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 strain *E. gergoviae* 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 hydrolyse 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 hydrolysed 96% of the methyl paraben within 15 minutes of inoculation, while in the same period of time, strain G1 hydrolysed 15%, and strain G12, 3.5% of the paraben. After 120 minutes, strain EM had completely hydrolysed 800 mg L^{-1} of methyl paraben, while strain G1 hydrolysed 92%, and strain G12, 55% of the paraben. The *B. cepacia* IFO strain did not substantially hydrolyse 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 Touch-down 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 hydrolyses 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 hydrolyse 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-free extracts of strain EM to hydrolyse 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 hydrolyse the same amount of methyl paraben as the *E. cloacae* strain EM, in two hours. The second strain of *E. gergoviae* (G12) also contained a homologue of *prbA*. However, its MIC was similar to the other *E. gergoviae* strains, with the exception of strain G1. The fact that it did not show an increased resistance towards paraben could be explained by the fact that its hydrolytic activity was smaller than EM and G1 (Figure 4). Previous

reports have indicated a MIC for *Enterobacter cloacae* of 1000 mg L^{-1} of methyl paraben (8). We report MICs of methyl paraben approximately 3-fold 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 – 10^7 cell/mL vs. approximately 3 x 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 towards these compounds. A well-documented method of resistance in *Escherichia 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), raising the possibility that the remaining species tested may resist to the parabens by a mechanism of efflux. Nevertheless, the increased resistance towards methyl paraben of strain EM and G1 and of the *E. coli* DH5α strain containing the *prbA* gene, shows that a high hydrolytic activity increases the resistance toward methyl paraben, as shown by the increases in the MICs of these bacteria.

Analysis of the *prbA* gene from *E. cloacae* EM with the nucleotide database using the nucleotide-nucleotide BLAST program showed a conserved stretch of 23 nucleotides (1679 – 1701) corresponding to the sequence TACTGGACCAACTTTGCCAAAAC, translating to the amino acids YWTNFAK (447 – 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 (*H. sapiens*) and represent either bile-salt dependent lipases or cholesterol esterases, although several others were carboxyl lipases or cholesterol esterases from the rat (*R. norvegicus*), the gorilla (*G. gorilla*) or the rabbit (*O. cuniculus*). A similar alignment of the amino acid sequence of PrbA 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 these 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 *Deinococcus radiodurans*, a polyurethane esterase from *Delftia 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 analyses of the sequences of PrbA from *E. cloacae* EM and those of *E. gergoviae* G1 and G12 seem to indicate greater homology to a number of esterases of eukaryotic origin from animals which 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-hydrolysing 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 non-enteric species indicate either convergent evolution or alternatively, a more distant

origin for the PrbA esterase. As *Enterobacter* species are ubiquitous in nature, the presence of certain strains containing the esterase able to hydrolyse 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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FIGURE LEGENDS

Figure 1: Conserved amino acid region in type-B carboxylesterases. The amino acid sequences of representative carboxylesterases were aligned with the Pileup program. Dradiodurans: *Deinococcus radiodurans* (GenBank accession number: AAF12163 and AAF09993), Dacidovorans: *Delftia acidovorans* (BAA76305), Scoelicolor: *Streptomyces coelicolor* (CAB55678 and CAA22794), *Bacillus* BP-23 and BP-7 (CAB42083 and CAB93516), Mtuberculosis: *Mycobacterium tuberculosis* (CAA17259), Aoxydans: *Arthrobacter oxydans* (AAA22078), Mpersicae: *Myzus persicae* (CAA52649), Celegans: *Caenorhabditis elegans* (CAB03277). Amino acid numbering refers to the Dradiodurans1 sequence.

Figure 2: Cloning of the *prbA* gene. A) Amplification of the 300-bp N-terminus region of strain EM by Touch-down PCR, *a)* total chromosomal DNA, *b)* plasmid DNA; B) Hybridization of chromosomal fragments of strain EM generated by *a) Bam*HI, *b) Eco*RI, *c) Hin*dIII, *d) Pst*I 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.

Figure 3: Hydrolysis of 600 mg L^{-1} (4.0 mM) of methyl paraben (—) and production of *p*-hydroxybenzoic acid (----) by *E. coli* DH5α containing the *prbA* gene cloned in the pGEM-T Easy vector (◆/◇), by the *E. cloacae* strain EM (●/○), and by *E. coli* DH5α containing the pGEM-T Easy vector alone (■/□).

Figure 4: Paraben esterase activity in strains *E. cloacae* EM (○/●) and *E. gergoviae* G1 (◇/◆) and G12 (△/▲). The produced amount of the degradation product *p*-hydroxybenzoic acid (----) is consistent with the amount of methyl paraben (—) concurrently degraded.

Table 1: Origin of strains, minimum inhibitory concentrations and hydrolytic ability

Strain Designation	Origin	MIC (mg L^{-1}) [f]	Degradation (%) [g]
E. cloacae EM [a]	Mineral supplement	>3200	99.9
E. cloacae ID 67037 [b]	Clinical strain	3200	12.7
E. cloacae ID 67284 [b]	Clinical strain	3200	12.8
E. cloacae LSPQ 3022	Reference strain	3200	15.3
E. cloacae LSPQ 3345	Reference strain	3200	19.2
E. gergoviae G1 [c]	Eye liner	>3200	100
E. gergoviae G2 [c]	Foundation	3200	10.3
E. gergoviae G3 [c]	Exfoliating gel	3200	8.9
E. gergoviae G4 [c]	Foundation	3200	8.5
E. gergoviae G5 [c]	Clinical strain	3200	7.8
E. gergoviae G6 [c]	Cream	3200	10.4
E. gergoviae G7 [c]	Challenge Test	3200	14.7
E. gergoviae G8 [c]	Shampoo	3200	14.4
E. gergoviae G9 [c]	Cream	3200	11.4
E. gergoviae G10 [c]	Shampoo	3200	12.3
E. gergoviae G11 [c]	Cream	3200	16.3
E. gergoviae G12 [c]	Rinsing material	3200	97.9
E. gergoviae G13 [c]	Clinical strain	1600	15.5
E. gergoviae G14 [c]	Clinical strain	3200	20.0
E. gergoviae G15 [c]	Clinical strain	3200	22.6
E. gergoviae GM [a]	Mineral supplement	3200	18.7
E. gergoviae WL [d]	Syrup	3200	14.8
E. gergoviae LSPQ 3347	Reference strain	3200	14.0
E. gergoviae ATCC 33028	Reference strain	3200	12.6
E. gergoviae ATCC 33426	Reference strain	3200	14.7
E. gergoviae ATCC 33428	Reference strain	3200	18.1
E. aerogenes AE1 [c]	Clinical strain	3200	15.4
E. aerogenes AE1 [c]	Clinical strain	3200	12.7
E. aerogenes AE1 [c]	Clinical strain	3200	14.7
E. aerogenes LSPQ 3811	Reference strain	3200	15.6
E. aerogenes ATCC 13048	Reference strain	3200	12.9
P. agglomerans ID 60635 [b]	Clinical strain	3200	10.1
P. agglomerans ID 65518 [b]	Clinical strain	3200	14.4
P. agglomerans LSPQ 3353	Reference strain	1600	14.5
P. agglomerans LSPQ 3825	Reference strain	800	13.5
E. coli ID 41839 [e]	Clinical strain	3200	9.1
E. coli ATCC 25922	Reference strain	1600	12.6
E. coli ATCC 35218	Reference strain	3200	11.3
B. cepacia IFO 15124	Oil-in water	1600	37.9
P. aeruginosa ID - 33122 [e]	Clinical strain	3200	12.4
P. aeruginosa ATCC 33350	Reference strain	1600	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 Dr. C. Bollet, Faculty of Medicine, INSERM, Marseille, France.

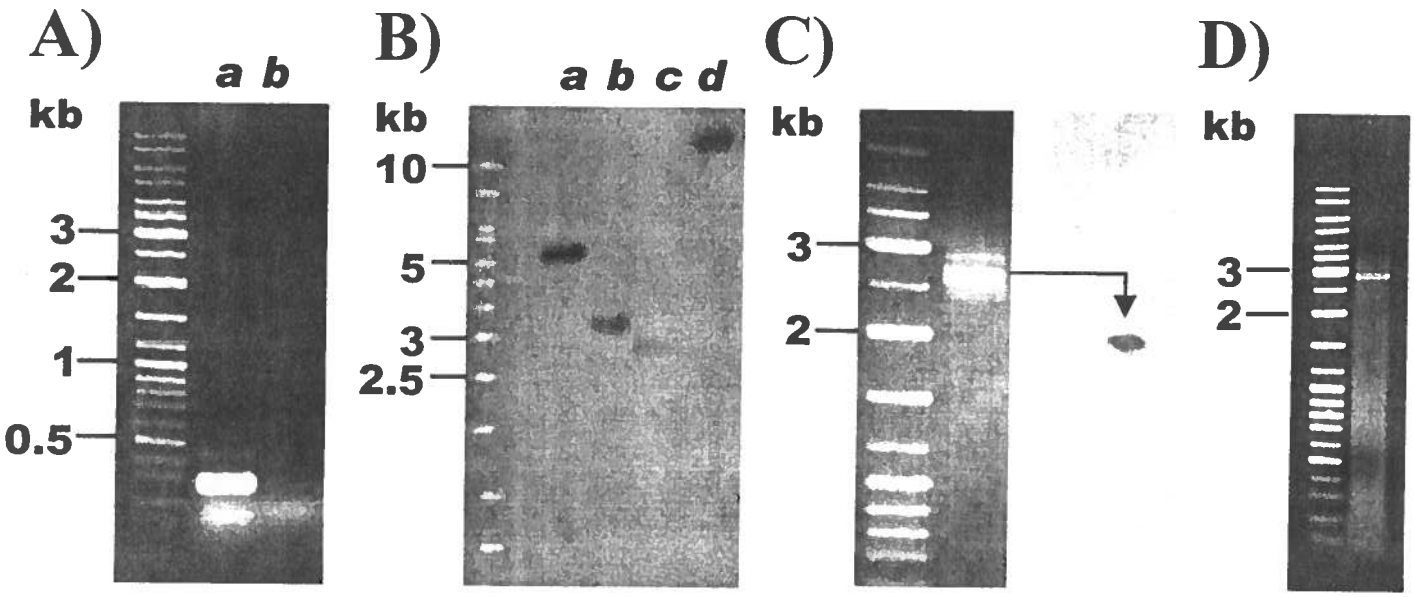
[d] Provided by a private company in Ontario, Canada.

[e] Provided by the Maisonneuve-Rosemont Hospital, Montreal, Canada.

[f] Expressed as concentration of methylparaben.

[g] Expressed as the percentage of 800 ppm (5.7 mM) of methyparaben degraded after 2 hours of incubation by an exponentially-growing culture.

```
                    129                                  152
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----
```


A)
a b
kb
3
2
1
0.5
B)
a b c d
kb
10
5
3
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C)
kb
3
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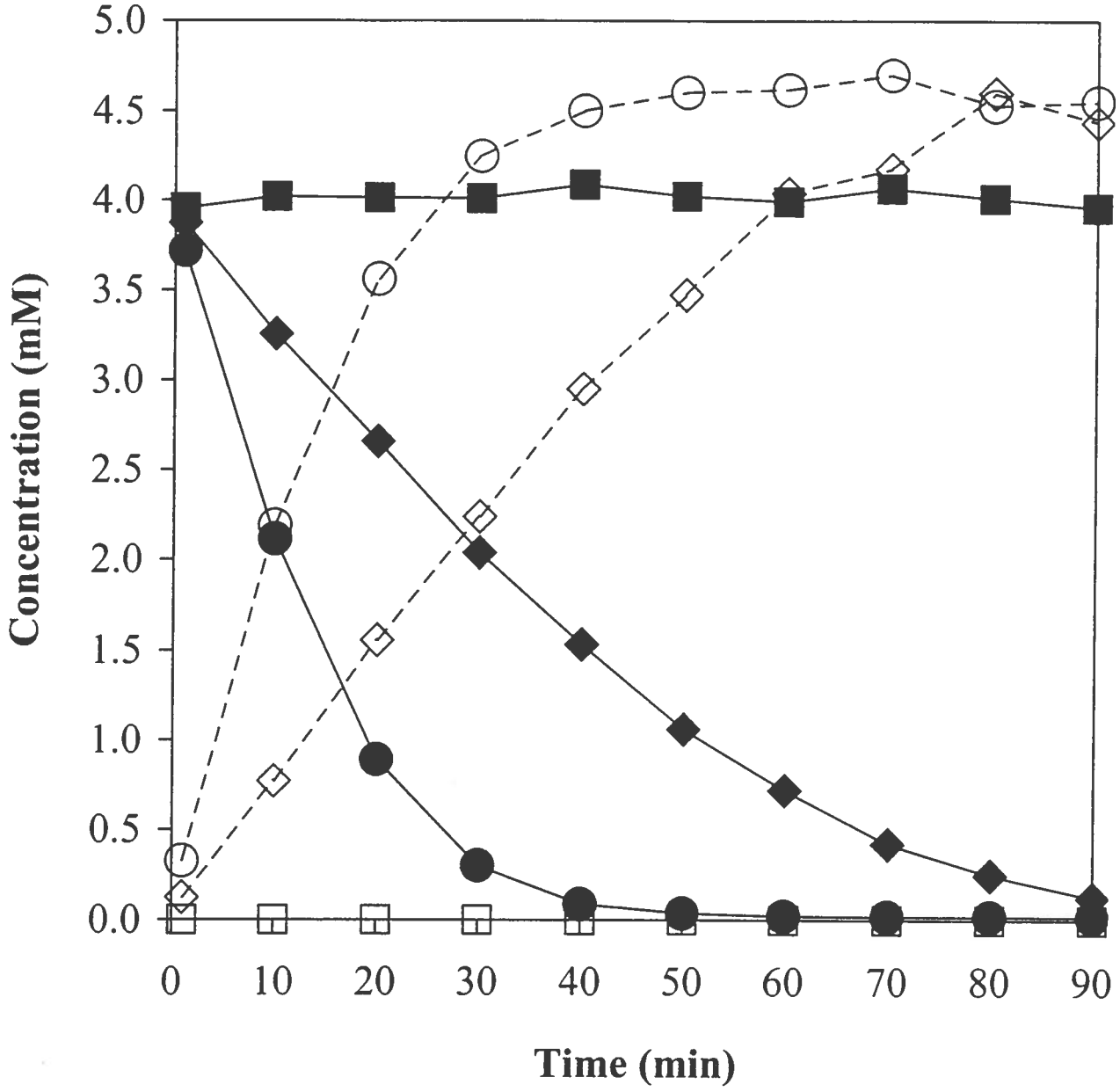
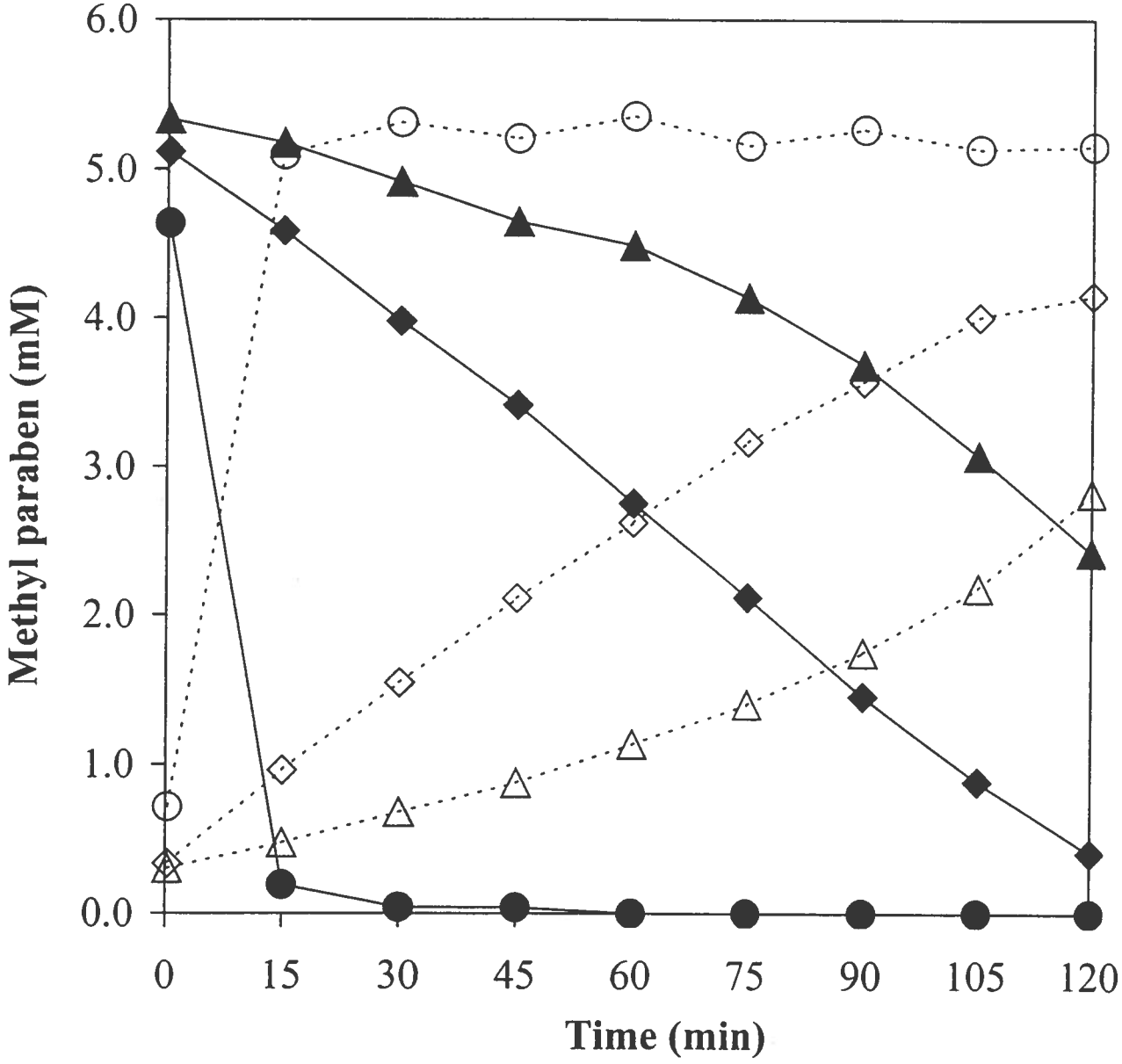
D)
kb
3
2


5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Concentration (mM)
0
10
20
30
40
50
60
70
80
90
Time (min)


6.0
5.0
4.0
3.0
2.0
1.0
0.0
Methyl paraben (mM)
0
15
30
45
60
75
90
105
120
Time (min)

Chapitre 3

L'estérase PrbA de la souche *E. cloacae* EM conférant un résistance accrue aux parabènes a été purifiée et caractérisée. Le protocole de purification a utilisé trois étapes de chromatographie en colonne et a résulté en une augmentation de l'activité spécifique de 60 fois relativement à un extrait sans cellules. Le poids moléculaire de l'enzyme purifiée était de 54.6 kDa. L'estérase a démontré une plus haute activité envers les parabènes à chaîne courte tels que le méthyle et éthyle parabène et une faible affinité pour l'acétate de *p*-nitrophényle. PrbA était capable d'hydrolyser plusieurs analogues structurels des parabènes, avec une haute activité envers le 3-hydroxybenzoate de méthyle, le 4-aminobenzoate de méthyle et le vanillate de méthyle. L'activité optimale de l'enzyme se situait à 31°C et à pH 7.0 et l'enzyme était active à plus de 90% de son activité entre 29 et 35°C et entre des valeurs de pH de 4.5 à 7.5. L'estérase était capable de transestérifier efficacement le méthyle et propyle parabène avec une concentration de 0.5% d'une série d'alcools allant du méthanol au *n*-butanol et aussi avec 5% de méthanol. L'activité estérasique était complétement inhibée par le diisopropylfluorophosphate (DFP), suggérant la participation d'une sérine au site actif. L'enzyme a aussi été complètement inactivée par le diéthylpyrocarbonate (DEPC), et une récupération partielle d'activité a été obtenue après l'addition d'hydroxylamine, suggérant la participation d'une histidine au site actif. De plus, les inhibiteurs 1-chloro-3-tosylamido-4-phényle-2-butanone (TPCK) et 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), qui sont spécifiques aux histidines ont causé une inhibition partielle de l'activité enzymatique et leur constante d'inhibition K_i a été mesurée à 290 et 200 μM, respectivement. Il a été déterminé que seulement une molécule de DFP ou de TLCK se liait à une molécule d'enzyme, suggérant la modification spécifique d'une seule sérine ou histidine, respectivement. La fragmentation en spectrométrie de masse du peptide tryptique contenant le site actif postulé a démontré deux séries d'ions y^{-2} et b^{+2} qui démontrent l'addition d'une molécule de DFP à Ser189, identifiant ainsi cet acide aminé comme la sérine catalytique.

PURIFICATION AND CHARACTERIZATION OF PrbA, A NEW ESTERASE FROM *ENTEROBACTER CLOACAE* HYDROLYZING THE ESTERS OF 4-HYDROXYBENZOIC ACID (PARABENS)

Nelly Valkova, François Lépine*, Louisette Labrie, Maryse Dupont and Réjean Beaudet.
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Running title: Characterization of the paraben esterase PrbA

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SUMMARY

The esterase PrbA from *Enterobacter cloacae* strain EM has previously been shown to confer additional resistance to the esters of 4-hydroxybenzoic acid (parabens) to two species of *Enterobacter*. The PrbA protein has been purified from *E. cloacae* strain EM using a three-step protocol resulting in a 60-fold increase in specific activity. The molecular weight of the mature enzyme was determined to be 54 619 ± 1 Da by mass spectrometry. It is highly active against a series of parabens with alkyl groups ranging from methyl to butyl, with $K_{m\ (app)}$ and $V_{max\ (app)}$ values ranging from 0.45 – 0.88 mM and 0.031 – 0.15 mM/min, respectively. The $K_{m\ (app)}$ and $V_{max\ (app)}$ values for *p*-nitrophenyl acetate were 3.7 mM and 0.051 mM/min. PrbA hydrolyzed a variety of structurally analogous compounds, with activities ≥ 20% relative to propyl paraben for methyl 3-hydroxybenzoate, methyl 4-aminobenzoate or methyl vanillate. The enzyme showed optimum activity at 31°C and at pH 7.0. PrbA was able to transesterify parabens with alcohols of increasing chain length from methanol to *n*-butanol, achieving 64% transesterification of 0.5 mM propyl paraben with 5% methanol within 2 hours. PrbA was inhibited by TPCK and TLCK, with K_i values of 0.29 and 0.20 mM, respectively, and was irreversibly inhibited by DFP or diethylpyrocarbonate. The stoichiometry of addition of DFP to the enzyme was 1:1 and only one TLCK molecule was found in the TLCK-modified enzyme, as measured by mass spectrometry. Analysis of the tryptic digest of the DFP-modified PrbA demonstrated that the addition of a DFP molecule occurred at Ser189, indicating the location of the active serine.

INTRODUCTION

The esters of *p*-hydroxybenzoic acid, commonly named parabens, are important preservative agents in the pharmaceutical, cosmetic and food industries. Parabens are active over a wide pH range (pH 4 – 8), are colorless, odorless, nonvolatile, stable, have a low acute and chronic toxicity and a broad spectrum of activity against molds, yeasts and bacteria (1). The antimicrobial activity of the parabens increases with increasing alkyl chain length, although limitations on the use of longer chain-length parabens are imposed by their solubility in aqueous media. Hence, the methyl, ethyl, propyl and butyl parabens are more commonly used in commercial formulations. Their anti-microbial effectiveness can be enhanced by combining two or more parabens in a formulation, with the total paraben concentration seldom exceeding 0.2 – 0.3 % (2, 3).

Resistance to parabens can lead to the survival and proliferation of microorganisms in commercial products that are normally well-stabilized with these antimicrobial agents. One mechanism of resistance of bacteria towards parabens is hydrolysis of their ester bond. There are many such reported cases of resistance to the parabens in the literature. A strain of *Cladosporium resinae* isolated from a pharmaceutical suspension containing 0.2% of methyl paraben was able to hydrolyze this paraben (4). A strain of *Pseudomonas aeruginosa* was able to grow and to degrade parabens in an antimicrobial preparation used in the formulation of eye drops containing a mixture of methyl and propyl paraben at a total concentration of 0.3% (5). The *P. aeruginosa* strain 396, isolated from an unpreserved oral formulation, was able to grow in the presence of 0.1 to 0.2% of methyl and propyl parabens and to hydrolyze propyl paraben to produce *p*-hydroxybenzoic acid (6). A strain of *Pseudomonas* (*Burkholderia*) *cepacia* isolated from an oil-in-water emulsion containing 0.1 – 0.2% methyl and propyl parabens was able to hydrolyze both parabens (7).

Although bacterial resistance to parabens has been reported in several species, there are few reports of specific enzymes with the ability to degrade parabens. The degradation of parabens by the resistant *P. aeruginosa* strain 396 was proposed to

proceed through inducible intra- and extracellular esterases, although these enzymes were not isolated (6). An esterase purified from *Aspergillus flavus* capable to hydrolyze depside esters was shown to be active against the methyl and ethyl parabens as part of its substrate specificity profile, although the microorganism's resistance to the parabens was not reported (8). Additionally, four different carboxylesterases capable of hydrolyzing parabens have been identified in human skin and subcutaneous fat tissues. Two such esterases from subcutaneous fat tissues were more active towards short-chain parabens while a third one from transformed keratinocytes was more active towards longer chain-length parabens such as butyl paraben (9).

A strain of *Enterobacter cloacae* was isolated from a contaminated mineral supplement formulation containing 1700 ppm (11.2 mM) and 180 ppm (1.0 mM) of methyl and propyl paraben, respectively. The strain, named EM, was shown to be resistant to parabens through the action of an esterase which hydrolyzed the parabens to *p*-hydroxybenzoic acid (10). Here, we report the purification and characterization of the PrbA esterase active against parabens and other analogues.

EXPERIMENTAL PROCEDURES

Materials. All growth media, including Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA) were from Difco Laboratories (Detroit, MI). The CM Sepharose Fast Flow was purchased from Pharmacia Biotech (Baie d'Urfé, Qc), and the QMA and SP-5W columns were from Waters (Milford, MA). The methyl, ethyl, propyl and butyl parabens, *p*-hydroxybenzoic acid, as well as 1-chloro-3-tosylamido-4-phenyl-2-butanone (TPCK), 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK), diethylpyrocarbonate and hydroxylamine, were purchased from Sigma (St. Louis, MO). All the paraben analogues, *p*-nitrophenyl acetate, *p*-nitrophenol and *n*-propanol were purchased from Aldrich (Milwaukee, WI). Methanol and ethanol were from EM Science (Gibbstown, NJ), and *n*-butanol from A & C American Chemicals (Ville St-Laurent, Qc). Diisopropylfluorophosphate (DFP) was obtained from Calbiochem-Novabiochem (LaJolla, CA). Sequencing-grade modified trypsin was obtained from Promega (Madison, WI). Solid-phase extraction of tryptic peptides was carried out on C4 or C18 Ziptips from Millipore (Bedford, MA). Type D nanoflow probe tips for mass spectrometry were from Micromass Canada (Pointe-Claire, Qc).

Purification of PrbA. A suspension of *E. cloacae* strain EM was inoculated at an approximate cell density of 1 x 10^7 cells/mL in minimal Davis medium containing 1% glucose. The cells were incubated overnight at 37°C, and were subsequently collected by centrifugation at 8000 rpm for 20 minutes and resuspended in 20 mM MES-NaOH buffer, pH 5.5. The cells were lysed by a three-fold French Press treatment at a pressure of 1200 psi, followed by a centrifugation step at 18 000 rpm for 45 minutes and two ultracentrifugation steps at 40 000 rpm for 90 minutes each. The cell-free protein suspension in 20 mM MES-NaOH buffer, pH 5.5 was filtered through a 0.2 µ membrane (Millipore, Bedford, MA) prior to application on the purification columns.

The first purification step used a CM Sepharose Fast Flow column with a 60:40 ratio of 20 mM MES-NaOH, pH 5.5, and 20 mM MES-NaOH containing 1 M NaCl for the first 30 minutes and followed by a gradient which reached 100% NaCl after 60

minutes, with a flow rate of 3 mL/min. Enzymatic activity was determined as previously described (10) and the presence of PrbA was confirmed by SDS-PAGE of the active fractions. The active fractions were pooled and dialyzed into 20 mM Tris·HCl buffer, pH 8.4. The second purification step, through a QMA column, used a gradient of 68:32 to 0:100 of 20 mM Tris·HCl, pH 8.4, and 20 mM Tris·HCl containing 1 M NaCl in 60 minutes at a flow rate of 2 mL/min. The active fractions were pooled and dialyzed into 20 mM MES-NaOH buffer, pH 5.5, and subsequently purified through a SP-5W column. The elution gradient was 100:0 to 30:70 of 20 mM MES-NaOH, pH 5.5, and 20 mM MES-NaOH containing 1 M NaCl within 60 minutes at a flow rate of 0.5 mL/min. The active fractions were subsequently pooled and stored at –70°C.

Substrate specificity of PrbA. All activity tests were done in 5.0 mL of 0.05 M sodium phosphate buffer, pH 6.8, containing the selected paraben at concentrations of 0.5 or 1.0 mM and at 30°C. The activity was measured after the addition of enzyme to a concentration of 0.2 nM over an assay time of 5 minutes. The hydrolysis of the parabens and the amount of *p*-hydroxybenzoic acid produced was quantified by HPLC according to the method described previously (10). The specific activities are expressed in millimoles of *p*-hydroxybenzoic acid produced per minute per milligram of protein (mmol/min·mg). Specific activity measurements were done in triplicate and are reported with the standard deviation. The $K_{m\ (app)}$ and $V_{max\ (app)}$ values for the parabens were determined using 10 sequential two-fold dilutions of 1.0 mM solutions of methyl and propyl paraben and of a 0.5 mM solution of butyl paraben in 0.05 M sodium phosphate buffer, pH 6.8. Due to limitations in substrate solubility, the precise K_m and V_{max} values could not be measured. All $K_{m\ (app)}$ and $V_{max\ (app)}$ values were calculated by non-linear regression analysis with the software EZ-Fit 5.0 (Perella Scientific, Amherst, NH).

The $K_{m\ (app)}$ and $V_{max\ (app)}$ measurements for *p*-nitrophenyl acetate were done in 2.0 mL of seven consecutive two-fold dilutions of a 1.0 mM solution in 0.05 M sodium phosphate buffer, pH 6.8, containing 4% acetone, to which 0.2 nM of enzyme were added. The enzyme activity was measured by monitoring the appearance of *p*-nitrophenol by absorbance readings at 405 nm. The *p*-nitrophenol produced was quantified based on

the calibration curve of a 1.0 mM *p*-nitrophenol standard in sodium phosphate buffer containing 4% acetone. The background hydrolysis rate of *p*-nitrophenyl acetate was subtracted from each assay.

All substrate analogues, including a reference solution of propyl paraben, were prepared at stock concentrations of 100 – 400 μM in 0.05 M of sodium phosphate buffer, pH 6.8, and were diluted to 50 μM. All assays with substrate analogues were done with 0.2 nM of enzyme as described above, with assay times ranging from 5 to 15 minutes. The enzyme activity was measured as the amount of substrate hydrolyzed as quantified by HPLC as described above.

Stability of PrbA. The thermal stability profile of PrbA was obtained by measuring the enzyme activity with 1.0 mM of propyl paraben according to the assay procedure above in 0.05 M sodium phosphate buffer at pH 6.8. The pH stability profile was obtained by measuring the enzyme activity at 30°C in three different buffer systems. A 0.05 M sodium phosphate buffer was used at pH 2.0 to 3.5, and at 6.0 to 7.5. A 0.05 M sodium citrate buffer was used at pH 4.0 to 5.5, and a 0.05 M sodium borate buffer was used at pH 8.0 to 9.5.

Transesterification activity. All transesterification assays were done according to the same procedure as the substrate specificity assays, using 0.5 mM of methyl or propyl paraben in 5.0 mL of 0.05 M sodium phosphate buffer, pH 6.8, containing either 0.5% of methanol (0.12 mM), ethanol (0.086 mM), *n*-propanol (0.067 mM) or *n*-butanol (0.055 mM), or 5% of methanol (1.2 mM) or ethanol (0.86 mM). All assays were done in triplicate.

Inhibition of PrbA. The measurement of enzyme activity after inhibition with DFP, TPCK, TLCK or diethylpyrocarbonate was done according to the same procedure as the substrate specificity assays, using 100 μM of propyl paraben. Each inhibitor, at a concentration of 100 μM, was added at the mid-point of the assay, or alternatively, the enzyme was pre-incubated with the inhibitor for 10 minutes at 30°C prior to the addition

of the paraben. Verification of the reversal of the diethylpyrocarbonate-induced inhibition by hydroxylamine was done by incubating the enzyme and inhibitor for 10 minutes at 30°C, followed by another 10-minute incubation with 200 μM of hydroxylamine, and the subsequent addition of the paraben. The inhibition constant K_i for TPCK and TLCK was calculated from the relative $K_{m\ (app)}$ values observed for propyl paraben in the absence of the inhibitors and in the presence of a fixed concentration of 100 μM of TPCK or TLCK.

The stoichiometry of the addition of DFP or TLCK to PrbA was verified by incubating 5.6 μM of PrbA in 0.05 M of sodium phosphate buffer, pH 7.0 with 100μM of DFP or 3 mM of TLCK at 30°C for 30 minutes. The modified enzyme was subsequently purified from the sodium phosphate buffer and excess inhibitor by solid-phase extraction with a C4 Ziptip using a 60:40 water:acetonitrile eluent with 0.1% acetic acid, to which 10% acetic acid was subsequently added. The molecular weight of the native and modified enzymes was calculated from several mass spectrometry measurements using a Quattro II Triple Quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) equipped with a Z spray interface in nanospray. The molecular weight measurements were done in positive electrospray ionization mode with a capillary voltage of 0.7 – 0.9 kVolts and a cone voltage of 35 – 45 V.

Chemical modification of Ser189. A concentration of 5.6 μM of PrbA in 0.05 M of sodium phosphate buffer, pH 7.0, was incubated with 100 μM of DFP at 30°C for 30 minutes. Subsequently, this mixture was added to 1 μg/μL of trypsin in an equivalent volume of 0.025 M of NH_4HCO_3 buffer, pH 8.2, for an overnight incubation at 37°C. The tryptic peptides were purified by solid-phase extraction with a C18 Ziptip and eluted with a 60:40 water:acetonitrile mixture containing 0.1% acetic acid. Subsequently, the concentration of acetic acid was increased to 10%. Collision-induced dissociation (CID) studies of the tryptic peptides were done with argon at 38 – 42 eV collision energy.

RESULTS

Purification of PrbA. Three steps on a cell-free lysate from *E. cloacae* strain EM were necessary to purify the esterase PrbA to homogeneity (Table 1). The first step, through a CM Sepharose column, resulted in a 7-fold increase in the specific activity of the enzyme. The second step, using a QMA column, was the most efficient in isolating PrbA, as the specific activity after this step increased 55-fold relative to the crude extract. The final step, with a SP-5W column, purified the enzyme to homogeneity and slightly increased the specific activity, reaching 60-fold relative to the activity of the initial cell-free extract. The enzyme was purified to homogeneity, as determined by SDS-PAGE stained by the silver nitrate method, and its molecular weight was calculated at 54.6 kDa. The molecular weight of the enzyme was 54 619 ± 1 Da as measured by mass spectrometry.

Substrate specificity of PrbA. The specific activities of the purified enzyme were determined with the most commonly used series of parabens, from methyl to butyl paraben, and are shown in Table 2. The specific activity of PrbA is greatest with ethyl paraben, reaching 2.71 mmol/min·mg. The specific activity towards methyl paraben is nearly as high, reaching 91% of that with ethyl paraben. The activity declines with longer chain length parabens, reaching 70% activity with propyl paraben and 35% activity with butyl paraben.

The $K_{m\ (app)}$ and $V_{max\ (app)}$ values were determined for methyl paraben and for the longer chain-length propyl and butyl parabens (Table 2). The $K_{m\ (app)}$ values decrease approximately 2-fold as the alkyl chain length increases, from 0.88 mM with methyl paraben to 0.45 mM with butyl paraben. The $V_{max\ (app)}$ values decrease approximately 5-fold with increasing chain length, from 0.15 mM/min with methyl paraben to 0.031 mM/min with butyl paraben. The $K_{m\ (app)}$ value for *p*-nitrophenyl acetate is approximately 4-fold greater than that obtained with methyl paraben, while the $V_{max\ (app)}$ value for *p*-nitrophenyl acetate is closest to the value obtained with butyl paraben.

The activity of PrbA towards several structural analogues of the parabens was determined relative to the activity with propyl paraben (Table 3). The enzyme activity was highest with propyl paraben, although the activity with methyl 3-hydroxybenzoate was nearly as high, reaching 97%. The second highest activity was obtained with methyl 4-aminobenzoate, at 21%, and with methyl vanillate at 22%. Similar activities, reaching 12%, were obtained with methyl 3,5-dihydroxybenzoate and ethyl 3,4-dihydroxybenzoate. The activity with the remaining substrates, methyl benzoate, methyl 2-aminobenzoate, methyl 2-hydroxybenzoate and *trans*-methyl cinnamate ranged from 3 to 6%. PrbA was not able to hydrolyze the ester bond of *tert*-butyl paraben nor the amide bond of 4-hydroxybenzamide.

Stability of PrbA. The activity profile of PrbA at different temperatures shows an optimum activity at 31°C and ≥ 90% activity between the temperatures of 29 – 35°C. The enzyme retained ≥ 80% of its activity between 25 and 45°C, and more than 50% activity at 50°C and 15°. The activity of PrbA at different pH values is greatest at pH 7.0 and the enzyme maintains ≥ 90% of its activity in the pH range 4.5 – 7.5. The enzyme retains approximately 70 – 80% of its activity at pH values of 4.0 and 8.0. The activity decreases significantly outside of this range, reaching 11% at pH 3.0 and 4% at pH 9.5. The enzyme is completely inactivated at pH 2.5.

Transesterification activity. PrbA transesterified methyl or propyl parabens with 0.5% (v/v) of methanol, ethanol, *n*-propanol and *n*-butanol (Table 4). The rate of disappearance of propyl paraben was 7% higher with methanol than in the buffer alone (1.73 and 1.61 mmol/min·mg, respectively), while the rate of formation of *p*-hydroxybenzoic acid was 60% slower with methanol than without (0.617 and 1.52 mmol/min·mg, respectively). The rate of disappearance of methyl paraben (1.56 mmol/min·mg) was reduced by 23% in the presence of ethanol, and by 60% and 77% with *n*-propanol and *n*-butanol, respectively, which can be partially explained by the decrease in the molar concentration of alcohols present. The rate of formation of *p*-hydroxybenzoic acid in the presence of either of these three alcohols ranged from 17 to 43% of that without any alcohol (1.75 mmol/min·mg). The rate of formation of the corresponding transesterified paraben was

approximately 50% of the rate of disappearance of the original paraben with methanol, ethanol or *n*-propanol, and reached only 14% with *n*-butanol.

Additionally, PrbA was able to carry out the efficient transesterification of propyl paraben with 5% v/v (1.2 mM) of methanol (Fig. 1). The yield of methyl paraben produced after 2 hours was 64% of the initial amount of propyl paraben in solution (0.5 mM), while at the same time, the yield of the side product *p*-hydroxybenzoic acid was minimized to 8% of the amount of propyl paraben initially present. The yield of the transesterified paraben was reproducible, as shown by the low margin of error (Fig. 1). The same transesterification reaction with 5% v/v (0.86 mM) of ethanol was much less rapid under the same conditions. After 2 hours, only 13% of the initial propyl paraben was transformed, yielding 10% of ethyl paraben and 2% of *p*-hydroxybenzoic acid relative to the original amount of propyl paraben (results not shown).

Inhibition of PrbA. Total inhibition of PrbA activity occurred when 100 μM of DFP were added at the mid-point of a hydrolysis assay with 100 μM of propyl paraben. Complete enzyme inhibition was also obtained under similar conditions by the addition of 100 μM of diethylpyrocarbonate and a 50% restoration of activity was measured after the addition of 200 μM of hydroxylamine to the diethylpyrocarbonate-modified enzyme (results not shown). The addition of 100 μM of TPCK or TLCK under comparable conditions resulted in a 64% and 68% enzyme inhibition, respectively. Since the addition of TPCK or TLCK resulted in only a partial enzyme inhibition, these compounds were characterized as possibly acting as reversible inhibitors, as suggested in a previous study on human serum butyrylcholinesterase (Cengiz *et al.*, 1997). The inhibition constant K_i for TPCK and TLCK was determined as 292 and 202 μM, respectively. The addition of TPCK or TLCK increased the $K_{m\ (app)}$ value of PrbA towards propyl paraben by 30 – 40%, and had only a slight impact on the $V_{max\ (app)}$ value, suggesting that both of these compounds could act as reversible competitive inhibitors of PrbA (results not shown).

The deconvoluted mass spectrum of PrbA after modification with DFP showed only one peak at 54 784 ± 1 Da (results not shown). This 164 Da mass increase

corresponds to the addition of one DFP molecule and the loss of 19 Da from the fluoride leaving group. The single addition of 164 Da confirmed a 1:1 stoichiometry of DFP addition to the enzyme. The DFP-modified PrbA was totally inhibited.

The deconvoluted mass spectrum of TLCK-modified PrbA presented the peak of the native enzyme at 54 619 ± 1 Da along with another at 54 948 ± 3 Da with a 17 % intensitiy of the initial peak (results not shown). This mass increase corresponds, within the experimental error, to the addition of one TLCK molecule, with the loss of 35 Da from the chloride leaving group. No other peak was observed corresponding to a multiple addition of TLCK. Only partial modification of PrbA could be achieved at 3 mM of TLCK, as higher concentrations of TLCK had a suppressing effect on the electrospray ionisation of the enzyme. This chemical modification resulted in a partial inhibition of enzyme activity, reducing the activity by 51%.

Chemical modification of Ser189. The addition of one DFP molecule to PrbA made the identification of the modified serine possible through collision-induced dissociation studies of the tryptic peptide containing the active site motif G-E-S-A-G-G conserved in many esterases. The full sequence of this tryptic peptide was NIQSFGGDNHNVTLFGESAGGHSVLAQMASPGAK (amino acids 172 – 205) with a molecular weight of 3399.7 Da. An ion corresponding to the $[M+4H]^{+4}$ charged state of this peptide was identified at m/z 851, and a low-intensity ion corresponding to the $[M+3H]^{+3}$ charged state was found at m/z 1134 (Fig. 2A). The ion at m/z 851 was confirmed to represent the active site peptide by collision-induced dissociation (CID), where the $y6^{+1} - y11^{+1}$, the $y13^{+2} - y24^{+2}$, and the $y26^{+3} - y33^{+3}$ ions were identified, spanning nearly the entirety of the peptide sequence as follows: IQSFGGDN(–)NVTLFGESAGGH(–)VLAQMA. Additionally, the $b20^{+2} - b21^{+2}$, and the $b15^{+3} - b17^{+3}$ ions, corresponding to the sequence FGE(–)(–)GG, were observed.

The peak at m/z 851 present in the tryptic digest of the native PrbA also represented two other peptides, which were identified by collision-induced dissociation. These peptides did not contain the serine active site motif. The first peptide, in a

$[M+2H]^{+2}$ charged state, contained the sequence LSILMVDYWTNFAK (amino acids 440 – 453) and had a molecular weight of 1699.9 Da. This peptide was identified from the series of $y5^+ - y13^+$ and $b5^+ - b9^+$ fragment ions. The second peptide, in a $[M+3]^{+3}$ charged state, contained the sequence KLPVMVWIPGGGLSSGSGNEYDASK (amino acids 97 – 121) and had a molecular weight of 2549.9 Da. This peptide was identified from the series of $y5^+ - y17^+$ and $b5^+ - b9^+$ fragmentation ions.

Upon modification by DFP, an ion at m/z 892 appeared, which was not present in the tryptic digest of the native enzyme (Fig. 2B). This ion corresponds to the molecular weight of the active site peptide with the addition of 164 Da from the DFP molecule, corresponding to 3563.7 Da, in the $[M+4H]^{+4}$ charged state. The $[M+3H]^{+3}$ ion, at m/z 1189, was also observed at a low intensity. The charged state of the m/z 892 ion was +4, as confirmed by the 0.25 Da separation of its isotopic peaks. The remaining ion at m/z 851 in the DFP-modified tryptic digest (Fig. 2B) is due to the presence of the two unrelated peptides described above.

The tryptic digests of the native and DFP-modified PrbA permitted the identification of a considerable number of peptides besides the active site peptide. A total of 24 peptides were identified from the native PrbA, out of a theoretical total of 42 tryptic peptides. An additional 5 peptides were identified from tryptic fragments which contained one missed cleavage site, covering 70 % of the entire enzyme. In the DFP-modified PrbA, the total number of identified peptides was 23, with an additional 11 peptides identified from fragments with one missed cleavage site, covering 81 % of the enzyme. Generally, the same peptides were identified from the native and DFP-modified enzymes, although their relative intensities were different (Fig. 2).

Collision-induced dissociation of the m/z 892 ion from the DFP-modified PrbA confirmed that it contained an active serine residue involved in the catalytic site. Although the entire peptide contained four serine residues which DFP could possibly modify, the serine of interest in the G-E-S-A-G-G motif was Ser189. In the collision-induced dissociation profile of this peptide, fragmentation at Ser189 corresponds to the

y17 ion or b18 ion (Fig. 3A). The CID spectrum of m/z 892 showed the $y12^{+2}$ – $y24^{+2}$ ions corresponding to the sequence NVTLFGESAGGH, with a mass addition of 164 Da from the $y17^{+2}$ (Ser) to the $y12^{+2}$ (His) ions, confirming the addition of 164 Da to Ser189 at m/z 866 (Fig. 3B). The $y20^{+2}$ ion (m/z 1038) is not indicated, as its signal is masked by the intense $b18^{+2}$ ion at m/z 1040. As well, the $b11^{+2}$ – $b18^{+2}$ ions corresponding to the sequence VTLF(G)ES were identified, as well as the $b23^{+2}$ and $b24^{+2}$ ions, which had an added mass of 164. The missing $b16^{+2}$ ion and several other ions between $b18^{+2}$ and $b23^{+2}$ correspond to the loss of glycine residues, which are often difficult to detect. In addition, the series of $y5^{+1}$ – $y17^{+1}$ ions corresponding to the sequence SAGGHSVLAQMA were identified, with a low intensity signal at m/z 1733 for the $y17^{+1}$ ion (Ser189 + 164 Da) (results not shown). The shift in mass at Ser189 by 164 Da identifies the site of DFP modification, and the concurrent total loss of activity confirms that Ser189 is the active serine in the catalytic site.

DISCUSSION

The esterase PrbA was purified from the paraben-resistant strain of *Enterobacter cloacae* EM. The molecular weight of the purified enzyme, as measured by SDS-PAGE, was 55 kDa. This measurement was confirmed by the theoretical molecular weight of the enzyme, calculated from the sequence of amino acids, deposited under the GenBank accession no. AAL82802, as 54 597 Da (11). The molecular weight of the enzyme as measured by mass spectrometry was 54 619 ± 1 Da, which corresponds to the addition of 22-23 mass units, possibly from a sodium ion originating from the sodium phosphate buffer. The esterase showed the highest specific activities towards the ethyl, methyl and propyl parabens, respectively, indicating a preference for shorter-chain esters (Table 2). Although the decreasing $K_{m\ (app)}$ values with increasing alkyl chain length indicating a higher affinity for the longer chain-length parabens, the higher $V_{max\ (app)}$ values with methyl paraben indicate that the rate of reaction is greatest with shorter chain-length parabens. The affinity of the enzyme for *p*-nitrophenyl acetate is relatively low, as indicated by a $K_{m\ (app)}$ value 4-fold higher than that for methyl paraben, as well as by a low $V_{max\ (app)}$ value.

PrbA showed an almost equal activity towards methyl 3-hydroxybenzoate as for propyl paraben (Table 3). However, the activity with methyl 2-hydroxybenzoate was only 3% of that with propyl paraben, indicating that the hydroxyl group must be placed in the *meta* or *para* position, but not in the *ortho* position, for efficient hydrolysis by PrbA. The presence of two hydroxyl or methoxy group substituents in the *para* and/or *meta* position still results in 12 to 22% residual activity. The absence of any polar substituent at the *para* or *meta* position leads to only 6% residual activity, as shown with methyl benzoate. The hydroxyl groups at the *para* position can be substituted by another electron-donating group such as an amine function, as with methyl 4-aminobenzoate, and PrbA still retained 21% of its activity. The analogues methyl 2-hydroxybenzoate and methyl 2-aminobenzoate showed a much reduced reactivity, confirming that the presence of a substituent at the *ortho* position results in significant loss of enzymatic activity. Interestingly, PrbA hydrolyzed methyl cinnamate, although in low yields, showing that

its activity was not limited only to benzoic ester analogues, as also shown by its activity against *para*-nitrophenyl acetate. PrbA could not cleave the ester bond of *tert*-butyl paraben, due to the steric hindrance at the carbonyl caused by the bulky *t*-butyl group, but utilization of this paraben is hampered by its very limited solubility in water. The activity of PrbA seems to be limited to ester bonds, as it could not cleave the amide bond of the structurally related 4-hydroxybenzamide.

It has been previously shown that a *B. cepacia* strain that could hydrolyze low concentrations of parabens (100 mg/L) in three weeks, could also perform the transesterification of ethyl paraben with various alcohols (12). This transesterification was attributed to an esterase, probably the same that hydrolyzed the paraben. However, the enzyme responsible was not isolated and the transesterification process, although efficient, required 3 days for 80% conversion of ethyl paraben to methyl paraben with 0.4% of methanol (12). The ability of PrbA to carry out paraben transesterification was then investigated with a series of alcohols of increasing chain length ranging from methanol to *n*-butanol. The efficiency and yield of transesterification was greatest with methanol and ethanol (Table 4), and decreased approximately by half with *n*-propanol. The yield of butyl paraben obtained upon transesterification with *n*-butanol was the lowest, reaching only 6% of the yield obtained with methanol. Furthermore, PrbA can carry out efficient transesterification with methanol concentrations as high as 5% within a short period of time (Fig. 1), achieving 64% transesterification in two hours.

The residues involved in the active site of PrbA were investigated. The catalytic site of many esterases contains a catalytic triad composed of an active serine, histidine and aspartic acid. The presence in the catalytic site of the serine and histidine residues was investigated using reagents known to react selectively with these active residues. The protease inhibitor DFP is known to react selectively with active serine residues (13). Modification of PrbA with DFP resulted in a complete irreversible inhibition with a 1:1 stoichiometry as observed by mass spectrometry. Analysis of the tryptic peptides of the DFP-modified PrbA showed that the modified residue was Ser189. This serine is part of the sequence G-E-S-A-G-G, which corresponds to the conserved G-X-S-X-G motif

commonly found in carboxylesterases (14), further confirming the identity of this serine as part of the active-site of PrbA. An alignment of the 100 esterases most closely related to PrbA with the protein-protein BLAST engine showed that this motif was conserved among all these esterases.

The protease inhibitor TLCK reacts selectively with active histidine residues (15). Only one molecule of TLCK was found in the modified enzyme. This suggests that the histidine residue that reacted was part of a catalytic triad. At the TLCK concentration used, only 17% of the enzyme was modified, as observed by mass spectrometry, while the activity was reduced by 51%. This difference could be attributed to the relative instability of the TLCK-derivatized histidine, especially at the low pH required (10% acetic acid) for efficient ionization in electrospray. The protein-protein BLAST alignment of the 100 esterases more closely related to PrbA showed that one residue, His412, was conserved in 98 out of the 100 proteins. His412 was also aligned with the regions of the other esterases containing the motif G-D-H-X-D. This motif is commonly found for the histidine in the catalytic triad of serine hydrolases (14), although it was not present within the sequence of PrbA itself.

A motif G-X-X-X-X-E-X-G, often indicative of the glutamate in the catalytic triad of serine hydrolases, was also located in the same BLAST alignment described above. This region included the highly-conserved Glu307 in PrbA, which was conserved in 98 out of the 100 esterases. The glutamate 307 in PrbA was part of a slightly-modified G-X-X-X-X-E-G motif, which was also found in several other homologous esterases instead of the G-X-X-X-X-E-X-G motif.

A small number of esterases have previously been shown to have the ability to hydrolyze parabens, namely from *P. cepacia*, *P. aeruginosa* or from human skin and subcutaneous fat tissue, as well as an esterase hydrolyzing depside esters from *A. flavus* (6, 7, 8, 9). However, none of these enzymes have been isolated or characterized in their purified form and hence, their amino acid sequence and other biophysical parameters are unknown. The esterase PrbA represents the first fully characterized carboxylesterase

responsible for the hydrolysis of parabens and it was shown that the *E. cloacae* strain EM containing the *prbA* gene was more resistant towards parabens than a *E. cloacae* reference strain that did not have a paraben-hydrolyzing activity (11). PrbA is most active at physiological pH and at a temperature near 30°C, which are conditions that often apply to commercial preparations containing parabens. Two *prbA* genes closely homologous to the *prbA* gene found in *E. cloacae* strain EM have been identified in two *Enterobacter gergoviae* strains recently isolated in France (11). Both strains hydrolyzed parabens and one of them was more paraben-resistant than a *E. gergoviae* reference strain. This geographical separation might indicate that homologues of the *prbA* gene are more widespread than currently known. Hence, the characterization of PrbA is an important step in understanding and limiting the growth of paraben-resistant microorganisms in commercial formulations.

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FIGURE LEGENDS

Figure 1: Transesterification profile of 0.5 mM of propyl paraben (O) with 5% methanol, showing the nearly complete disappearance of the paraben within two hours and the main product, the transesterified methyl paraben (◊), with a low yield of the hydrolysis product *p*-hydroxybenzoic acid (Δ). The error bars represent the standard deviation of triplicate measurements.

Figure 2: Mass spectrum of a tryptic digest of the native (A) and DFP-modified (B) PrbA. The ion at m/z 851 in the native protein represents the +4 charged state of the peptide containing the active serine. Upon DFP modification, an ion at m/z 892 appears, which corresponds to the same peptide with the addition of 164 Da, in the +4 charged state. The additional ions from the native and DFP-modified peptide in the +3 charged state are also observed.

Figure 3: Collision-induced dissociation of the active site peptide from the DFP-modified PrbA. A) Fragmentation profile of the entire peptide, showing the series of b-ions generated from the N-terminus and the y-ions from the C-terminus. B) CID spectrum showing the series of y^{-2} and b^{+2} ions characteristic of the active site peptide with the addition of 164 Da to Ser189, which corresponds to the y17 and b18 ion.

Table 1: Specific activity of PrbA after each purification step

Purification Step	Total Enzyme Activity [a] (μmol/min)	Total Protein [b] (mg)	Specific Activity (μmol/mg·min)	Purification Fold
Cell-free extract	3.5×10^6	173	2.0×10^4	1.0
CM Sepharose	1.7×10^6	11.7	1.4×10^5	7.0
QMA	7.5×10^5	0.714	1.1×10^6	55
SP-5W	2.5×10^5	0.216	1.2×10^6	60

[a] All kinetic assays were done with propyl paraben.

[b] The protein concentration was measured by the Folin-Lowry assay.

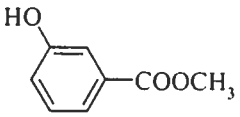
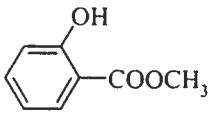
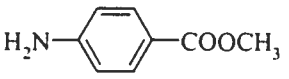
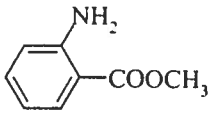
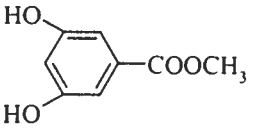
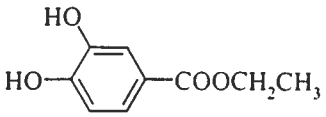
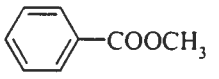
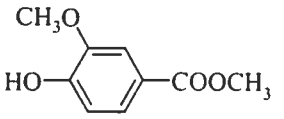
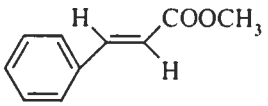
Table 2: PrbA activities towards the methyl to butyl parabens and *p*-nitrophenyl acetate

Substrate	Specific activity (mmol/min·mg)	$K_{m\ (app)}$ (mM)	$V_{max\ (app)}$ (mM/min)
Methyl paraben	2.46 ± 0.08	0.88	0.15
Ethyl paraben	2.71 ± 0.07	— [b]	— [b]
Propyl paraben	1.91 ± 0.03	0.60	0.078
Butyl paraben	0.942 ± 0.06	0.45	0.031
p-Nitrophenyl acetate	0.225 [a]	3.7	0.051

[a] Single determination.

[b] Not determined.

Table 3: Relative activity of PrbA towards paraben structural analogues

Substrate analogue	Chemical structure	% Activity [a]
Methyl 3-hydroxybenzoate		97
Methyl 2-hydroxybenzoate		3.0
Methyl 4-aminobenzoate		21
Methyl 2-aminobenzoate		6.1
Methyl 3,5-dihydroxybenzoate		12
Ethyl 3,4-dihydroxybenzoate		12
Methyl benzoate		6.1
Methyl vanillate		22
trans-Methyl cinnamate		3.6

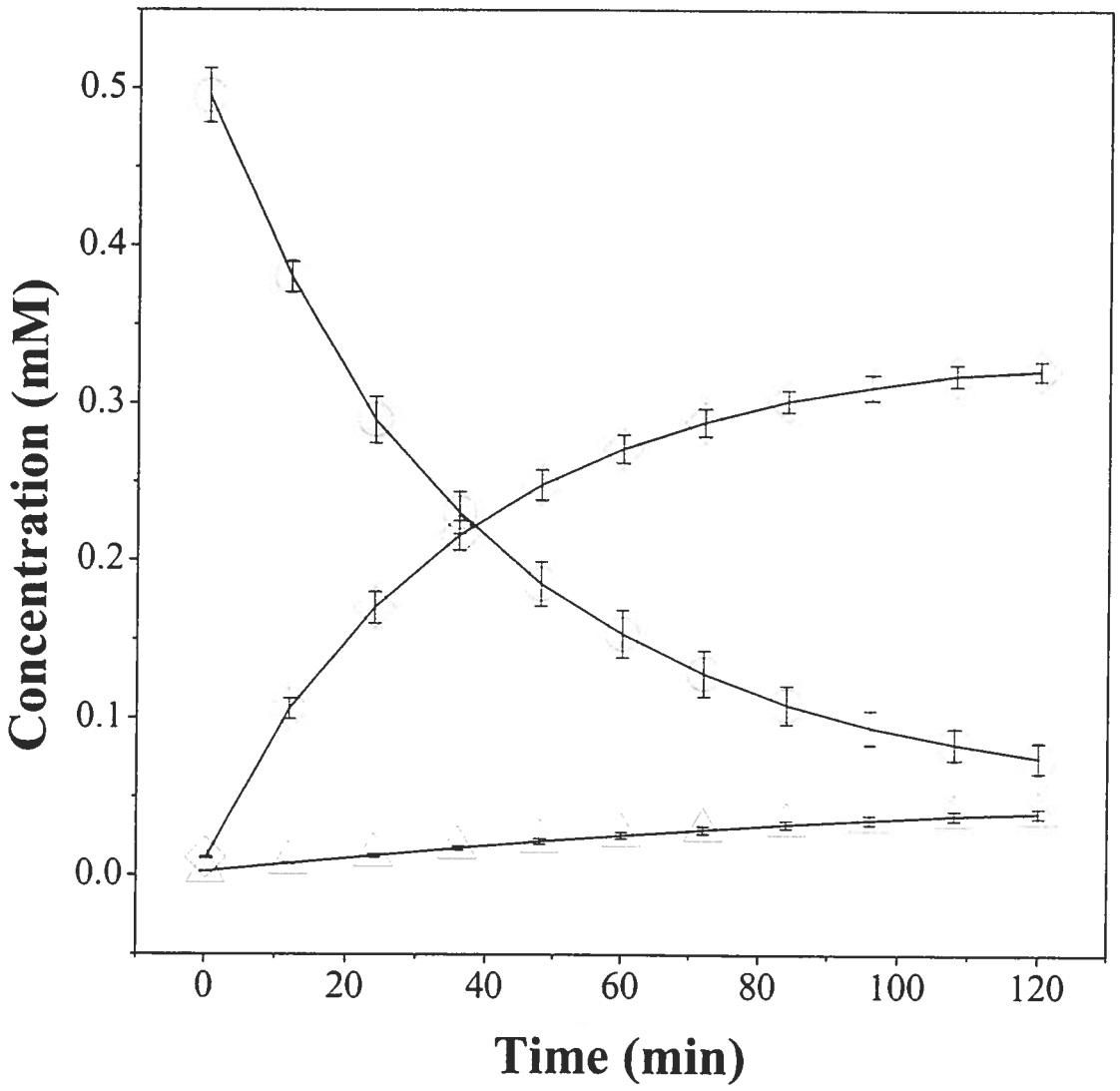
[a] The activity is expressed as a percentage of the activity obtained with propyl paraben.

Table 4: Rates of transesterification of methyl paraben with 0.5% ethanol, *n*-propanol and *n*-butanol and of propyl paraben with 0.5% methanol

0.5% Alcohol	Disappearance of original paraben (mmol/min·mg)	Formation of transesterified paraben (mmol/min·mg)	Formation of *p*-hydroxybenzoic acid (mmol/min·mg)
—[a]	1.56 ± 0.07	—	1.75 ± 0.08
—[b]	1.61 ± 0.06	—	1.52 ± 0.03
Methanol	1.73 ± 0.07	0.884 ± 0.06	0.617 ± 0.05
Ethanol	1.20 ± 0.03	0.672 ± 0.02	0.754 ± 0.03
n-Propanol	0.619 ± 0.04	0.303 ± 0.02	0.434 ± 0.02
n-Butanol	0.354 ± 0.03	0.0492 ± 0.003	0.303 ± 0.02

[a] Methyl paraben alone.

[b] Propyl paraben alone.


Concentration (mM)
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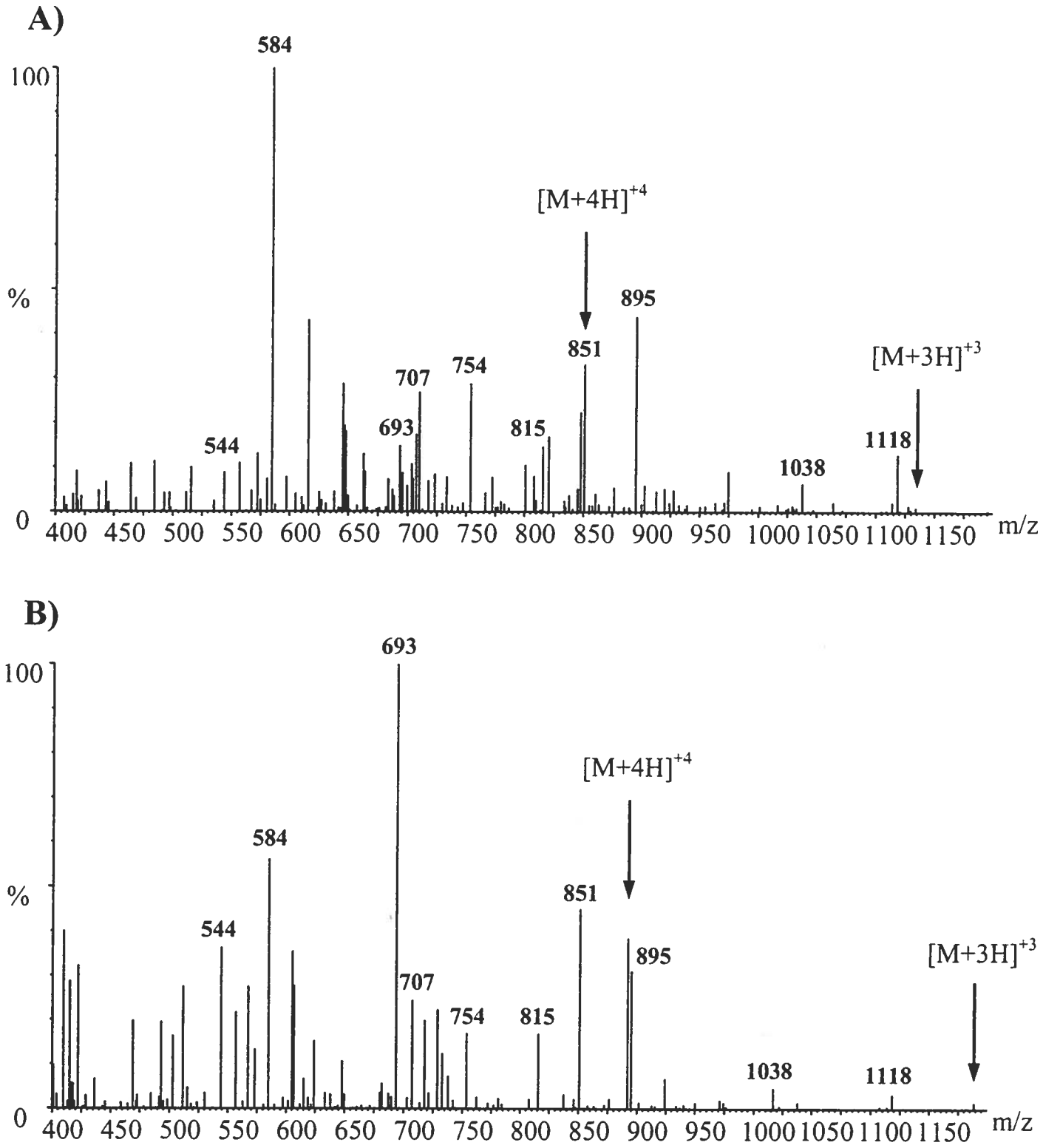
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
120
Time (min)


A)
584
100
[M+4H]+4
%
895
851
[M+3H]+3
707
754
693
815
544
1118
1038
0
400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150
m/z
B)
693
100
[M+4H]+4
584
%
851
544
895
[M+3H]+3
707
754
815
1038
1118
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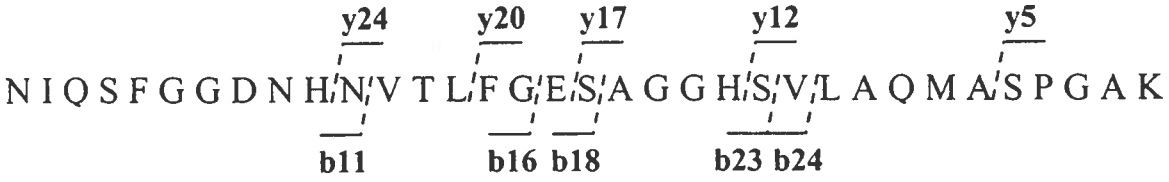
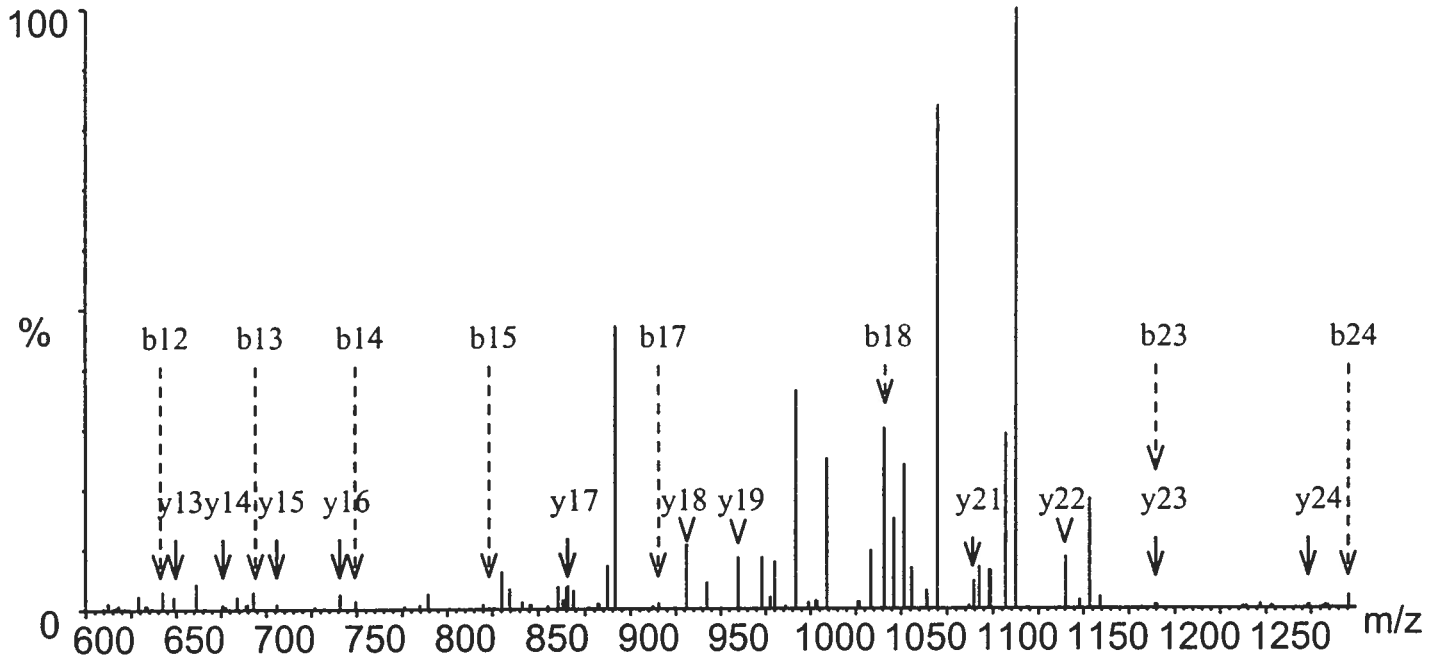
400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 1100 1150
m/z

A)


y24
y20
y17
y12
y5
N I Q S F G G D N H N V T L F G E S A G G H S V L A Q M A S P G A K
b11
b16 b18
b23 b24

B)


100
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b12
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600 650 700 750 800 850 900 950 1000 1050 1100 1150 1200 1250
m/z

Discussion

I. Synthèse des travaux

Une nouvelle enzyme responsable d'une augmentation de la résistance bactérienne aux parabènes a été identifiée et caractérisée. Cette enzyme, nommée PrbA, est une carboxylestérase de type B. L'estérase PrbA a été isolée d'une souche d'*E. cloacae* nommée EM contaminant un produit commercial stabilisé avec des parabènes. Il a été démontré que cette souche est capable d'hydrolyser les parabènes en acide 4-hydroxybenzoïque. De plus, une voie catabolique rarement observée de la transformation subséquente de l'acide 4-hydroxybenzoïque en phénol a été documentée. Le gène *prbA* responsable de la dégradation des parabènes chez la souche EM a été localisé sur l'ADN chromosomique et sa séquence a été déposée dans la collection GenBank. Deux souches d'*E. gergoviae* capables de transformer les parabènes en acide 4-hydroxybenzoïque ont démontré la présence d'un gène dont la partie N-terminale est fortement homologue à *prbA*. Ces deux souches ont été isolées de France, un endroit géographiquement éloigné du site d'isolement d'*E. cloacae* EM en Amérique du Nord, suggérant une répartition possiblement importante de ce gène. L'estérase purifiée était hautement active envers une série de parabènes allant du méthyle jusqu'au butyle, avec une préférence envers les parabènes à courte chaîne alkyle. L'estérase était active envers d'autres produits structurellement analogues aux parabènes qui contenaient des substituants hydroxyles aux positions autres que *para*, ainsi que des substituants amino ou methoxy. L'estérase était capable de transestérifier des parabènes avec une série d'alcools allant du méthanol jusqu'au *n*-butanol, avec un taux de conversion optimal obtenu avec le méthanol ou l'éthanol. L'activité de l'enzyme était inhibée par des composés modifiant spécifiquement les histidines, tels que le TPCK, TLCK et le DEPC, indiquant la participation possible d'une histidine catalytique. De plus, l'addition d'une seule molécule de DFP, qui est un inhibiteur spécifique aux sérines, a complètement inactivé l'enzyme, indiquant la participation d'une sérine dans le site catalytique. La sérine modifiée par le DFP a été identifiée comme Ser189 par fragmentation par spectrométrie de masse du peptide tryptique contenant le site actif postulé.

II. Avancement des connaissances

Une nouvelle enzyme capable d'hydrolyser les parabènes a été identifiée chez une souche de la bactérie ubiquitaire *E. cloacae*. Bien que des cas de résistance aux parabènes par des bactéries ont été décrits auparavant dans la littérature (Close et Nielsen, 1976; Hugo et Foster, 1964; Sokoloski *et al.*, 1962; Zedan et Serry, 1984), la présente étude a permis pour la première fois de proposer une voie métabolique complète des parabènes à partir de leur hydrolyse par l'estérase jusqu'à la production du produit de dégradation final, le phénol. La décarboxylation de l'acide 4-hydroxybenzoïque en phénol par la souche EM a été observée en conditions aérobies. Cette voie métabolique est rarement observée et a été documentée seulement une fois auparavant chez une entérobactérie (Grant et Patel, 1969; Patel et Grant, 1969). La présente étude confirme l'existence de ce mécanisme de décarboxylation pour supprimer un excès d'acide 4-hydroxybenzoïque chez les entérobactéries. Cette voie de transformation en conditions aérobies peut potentiellement prendre place dans des produits commerciaux stabilisés par des parabènes et contaminés par des entérobactéries comparables à la souche EM. Une telle contamination entraînerait la dégradation des parabènes et l'accumulation subséquente de phénol dans le produit, ce qui pourrait poser un risque pour la santé.

La séquence entière du gène *prbA* a été déposée dans la collection de GenBank et représente la première estérase conférant une résistance accrue aux parabènes à être séquencée. Le transfert du gène *prbA* à une souche d'*E. coli* sensible aux parabènes a resulté en une augmentation des CMI envers les parabènes ainsi qu'en l'acquisition d'une capacité hydrolytique comparable à celle de la souche EM. Ce résultat démontre que le transfert du gene *prbA* seul est suffisant pour acquérir la capacité de dégrader complètement les parabènes. La localisation chromosomique du gène *prbA* chez *E. cloacae* EM n'exclut pas la possibilité du transfert de ce gène à d'autres bactéries par contact direct, car ce gène pourrait faire partie d'un élément chromosomique mobile. Les travaux ci-dessus sont aussi les premiers à démontrer une distribution géographique étendue entre le gène codant pour cette parabène estérase présente dans des souches isolées de l'Amérique du Nord et de l'Europe. La présence de gènes homologues à *prbA*

a été démontrée chez deux souches d'*E. gergoviae* isolées en France, suggérant que ce gène de résistance aux parabènes n'est pas limité à une région géographique, et qu'il est possiblement répandu. De plus, la séquence de *prbA* démontre des homologies avec plusieurs carboxylestérases d'origine eucaryotique. Un nombre significatif d'estérases les plus identiques à *prbA* provenaient d'hôtes mammifères, tels que d'humain, de souris, rat, lapin, bovin ou porc. Ces hôtes contiennent tous un répertoire divers d'entérobactéries, suggérant la possibilité que le gène *prbA* a été originalement acquis par transfert horizontal.

Les travaux de caractérisation de l'estérase purifiée indiquent que PrbA est hautement active envers les parabènes et que son activité est optimale à des conditions environnementales applicables à plusieurs produits commerciaux. Ces paramètres cinétiques suggèrent que toute souche bactérienne exprimant ce gène pourrait dégrader les parabènes dans un produit sous conditions d'entreposage ou en utilisation courante et ainsi poser un danger de contamination. Les études sur *prbA* en présence de divers inhibiteurs ont permis de caractériser deux acides aminés impliqués dans le cycle catalytique de l'enzyme. Des études subséquentes sur le site catalytique viseraient l'identification de composés capables d'inhiber le site actif de l'estérase. Cette découverte aurait une application pratique, car un inhibiteur non-toxique pourrait être ajouté à des produits commerciaux stabilisés avec des parabènes afin d'inhiber l'action d'estérases similaires à PrbA. Les travaux de caractérisation du site actif suggèrent que PrbA contient probablement une triade catalytique caractéristique des carboxylestérases (Satoh et Hosokawa, 1998) et ainsi contribue aux connaissances générales sur la structure et les paramètres cinétiques des sérines estérases.

III. Contributions individuelles

Chapitre 1:

L'isolement de la souche d'*E. cloacae* EM du produit commercial contaminé, ainsi que son identification taxonomique par API et séquençage du rRNA 16S ont été accomplis préalablement et séparément des travaux de l'auteur. La détermination de la présence d'un plasmide chez *E. cloacae* a été faite également préalablement aux travaux de l'auteur. La démonstration de la dégradation des parabènes sur milieu solide contenant des parabènes crystallisés a été faite séparément des travaux de l'auteur. La quantification des parabènes et de leurs produits de dégradation a été faite selon une méthode de HPLC dévelopée au préalable avec des standards préparés par l'auteur. La confirmation de l'identité des produits de dégradation par GC-spectrométrie de masse a été faite selon une méthode d'analyse dévelopée au préalable.

Chapitre 2:

Une purification partielle de l'enzyme PrbA a été accomplie au préalable des travaux de l'auteur et a mené à une séquence de 20 acides aminés du N-terminal de l'estérase. Cette séquence a été utilisée pour construire un oligonucléotide dégénéré utilisé pour amplifier le gène *prbA*. Le deuxième oligonucléotide dégénéré est basé sur une région conservée retrouvée dans un alignement de la séquence d'acides aminés de plusieurs carboxylestérases construit préalablement aux travaux de l'auteur. Toutes les séquences ont été déterminées par le Service de Séquençage de l'INRS-IAF.

Chapitre 3:

Le protocole de purification de l'estérase PrbA a été developpé au préalable et séparément des travaux de l'auteur. L'estérase purifiée a été obtenue séparément des travaux de l'auteur.

Conclusion

La dégradation des parabènes par des bactéries résistantes peut mener à une contamination de produits commerciaux normalement bien stabilisés avec ces agents de conservation. Une telle souche contaminante d'*E. cloacae*, nommée souche EM, a été isolée. Le mécanisme de résistance de cette souche aux parabènes était inconnu lors de son isolement. Les travaux ci-dessus ont démontré que le mécanisme de résistance accrue d'*E. cloacae* EM consiste en une estérase d'une haute affinité pour les parabènes, localisée probablement dans le périplasme de la bactérie. La séquence de cette estérase a été établie, ainsi qu'un protocole pour sa purification. La séquence du gène *prbA* et la caractérisation de l'enzyme purifiée suggèrent que l'enzyme est une carboxylestérase, possiblement contenant une triade catalytique comprenant la sérine 189. Ces données représentent la première caractérisation complète d'une enzyme responsable de la résistance accrue aux parabènes chez une bactérie. De plus, elles suggèrent une distribution géographique étendue entre le gène découvert chez *E. cloacae* EM et un gène homologue présent chez des souches d'*E. gergoviae* isolées en France et suggèrent ainsi la possibilité du transfert de ce gène à d'autres bactéries qui peuvent potentiellement devenir des agents de contamination. Des recherches futures pouvant être envisagées incluent la détermination de la séquence de la région en amont du gène *prbA*, qui pourrait contenir un element mobile et ainsi fournir de l'appui à l'hypothèse du transfert horizontal du gène *prbA*. De plus, l'identification de l'histidine catalytique pourrait être accomplie en faisant des mutations ponctuelles sur PrbA et corrélant ces mutations avec le degré d'inactivation de la protéine.

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