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Resistance of a Strain of *Pseudomonas cepacia* to Esters of *p*-Hydroxybenzoic Acid

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Cells of a strain of *Pseudomonas cepacia* were isolated from an oil-in-water emulsion containing methyl and propyl *p*-hydroxybenzoates (methylparaben and propylparaben) as preservative additives. This strain demonstrated the ability to destroy these additives, to utilize the propyl ester as sole carbon source, and to hydrolyze the methyl ester. When the isolate was grown on Eugon agar, exposure to the methyl ester killed 99.9% of the inoculum, but the surviving cells grew logarithmically. On the other hand, cells grown on media containing propylparaben were less susceptible when subsequently exposed to emulsions containing methylparaben. These observations demonstrate one mechanism by which microorganisms develop resistance to antimicrobial preservatives.

Esters of *p*-hydroxybenzoic acid, commonly known as parabens, have been considered effective antimicrobial agents for the preservation of cosmetics (1, 4, 5, 9). However, Manowitz (7) reported that the contamination of presumably well-preserved formulations appears to be increasing. This is frequently due to the growth of microorganisms whose biochemical capabilities have been modified via mutation or selection and have thus become resistant to the toxic effects of conventional preservative systems. In this respect, members of the genus *Pseudomonas* are particularly versatile, being capable of utilizing a wide range of compounds as carbon and energy sources (11). This paper describes a propylparaben-resistant strain of *P. cepacia*, isolated from a preserved oil-in-water emulsion, which attacks paraben esters during growth in the presence of these compounds. In this degradation of the preservatives, disappearance of paraben esters parallels the appearance of *p*-hydroxybenzoic acid in experimental formulations. This strain is capable of developing resistance to the methyl ester and can cause its destruction, but it cannot use this compound as a carbon source. It can, however, utilize the propyl ester as a sole source of carbon and energy, under certain defined experimental conditions.

MATERIALS AND METHODS

Isolation and identification of a laboratory strain. During a routine screening procedure in our laboratory, an organism (strain 2487, also termed here "emulsion isolate") was isolated as a pure culture from plate counts in Eugon agar (BBL) of an emulsion formulation containing a mixture of methylparaben and propylparaben. The isolate was maintained on Eugon agar slants incubated at 35 C. The organism was tested for Gram reaction, motility, flagellar morphology, and characteristics on triple sugar iron agar (BBL). The biochemical reactions tested in the scheme adopted by our laboratory were completed using 24-h Eugon broth or agar cultures. The tests were incubated for 24 h at 35 C. A culture of *P. cepacia* (ATCC 17759) was obtained from the American Type Culture Collection and used as a control. Pigment production, cytochrome oxidase, lysine decarboxylase, gelatinase, arginine dihydrolase, citrate utilization, urease, oxidation/fermentation with glucose, arabinose, and lactose, and staining for the presence of intracellular fat were tested. Antibiotic sensitivity was tested with disks (Difco) using the following: gantrisin, 50 μg; gentamicin, 10 μg; chloramphenicol, 30 μg; polymyxin B, 50 U; colistin, 10 μg; and carbenicillin, 50 μg. Flagellar configuration was studied with a Hitachi model HV-125E electron microscope.

Growth of emulsion isolate on paraben media. Preliminary observations had shown that, although inocula of 10^5 organisms/g of Eugon agar cultures of this strain of *P. cepacia* were reduced to less than 1/g when plated immediately after inoculation of the emulsion containing methylparaben and propylparaben, the survivors rapidly overcame the inhibitory effect of the paraben esters and grew exponentially. To determine whether the organism could utilize paraben esters for growth, the following mineral salts medium was prepared: K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; NH_4NO_3, 0.1 g; granulated agar, 1.5 g; distilled water, 100.0 ml. To 100.0-ml portions of basal medium was added one of the following: (i) methylparaben, 0.1 g; (ii) propylparaben, 0.1 g; (iii) methylparaben, 0.1 g, plus propylpara-

ben, 0.1 g; (iv) glucose, 0.1 g; (v) methylparaben, 0.1 g, plus glucose, 0.1 g. Unsupplemented basal medium was used as a control. All media were sterilized by autoclaving at 121 C for 15 min. Growth from a 24-h slant of the isolate on Eugon agar was harvested by centrifugation, washed three times with sterile 0.85% saline, and resuspended in saline. A 1:10 dilution of this suspension was made in saline and delivered dropwise through Pasteur pipettes onto the hardened agar surfaces of media (i) through (v) and the control.

Growth of emulsion isolate in experimental formulations. To measure the preservative activity of paraben esters in experimental formulations, preservative testing was expanded to include challenge with the strain initially isolated from emulsions and subsequently cultured on propylparaben agar.

Inocula were prepared from 24-h slant cultures of (i) Eugon agar and (ii) mineral salts agar containing 0.1% propylparaben. Growth from the slant cultures was suspended in saline; cell suspensions were adjusted with saline as the blank to an optical density of 0.125 to 0.135 at 600 nm, using a Bausch & Lomb Spectronic 20 colorimeter.

The experimental samples challenged were: (i) emulsion with methylparaben, 0.17%; (ii) emulsion with propylparaben, 0.1%; (iii) emulsion with methylparaben, 0.17%, and propylparaben, 0.1%; (iv) emulsion without paraben. Each of these samples was inoculated with (i) approximately 340,000 cells/g grown on Eugon agar or (ii) 560,000 cells/g of cells grown on propylparaben agar. Platings were done at zero time and on days 1, 3, 6, 14, 21, and 28 in Trypticase soy agar with 0.07% lecithin and 0.5% polysorbate 80 (BBL). Plates were incubated at 35 C. Inoculated samples were stored at 25 C between platings.

Chromatographic assay of parabens. After the completion of growth pattern studies, all inoculated emulsion samples were assayed for paraben content. Uninoculated emulsion samples that had been stored at 4 C for 28 days served as controls. Individual paraben esters were analyzed by high-performance liquid chromatography, using a version of the classical column chromatography technique, modified to allow rapid separation and accurate quantitation of microgram levels of the components being assayed. This modification will be described in a forthcoming publication.

Samples were prepared by breaking the emulsion, adding a known weight of ethylparaben as an internal standard for quantitative analysis, and diluting with alcohol. A 5-μl amount of the alcoholic solution was injected onto the chromatographic column, which was then eluted by pumping a methanol-water (25:75, vol/vol) solution through the column. Ultraviolet absorbance of the eluate was continuously monitored with a single-wavelength (254 nm) photometer. As the individual parabens were eluted, a chromatographic trace was obtained and values were calculated for amounts of paraben esters remaining after storage of samples for 1 month at 25 C (see Table 1). The areas of methylparaben and propylparaben relative to ethylparaben in the samples and a standard were measured.

RESULTS

Characterization of emulsion isolate. The results of Gram staining, electron microscopy, and growth on triple sugar iron agar slants enabled us to classify the emulsion isolate as a nonfermentative, gram-negative pseudomonad which produced no soluble, visible, or fluorescent pigment and which exhibited polar multitrichous flagella.

Gelatin hydrolysis was slow, requiring 5 days for a positive reaction. The reactions were oxidative only for glucose, lactose, and arabinose. the emulsion isolate was sensitive to gantrisin, gentamicin, and chloramphenicol but resistant to polymyxin B, colistin, and carbenicillin.

The reactions of *P. cepacia* (ATCC 17759) were identical, except for the production of a yellow pigment by the ATCC strain and a beige-white coloration in the emulsion isolate.

Growth on paraben esters. When a mineral salts agar containing methylparaben, propylparaben, or else a combination of both esters was inoculated with washed suspensions of strain 2487 grown on Eugon agar, growth occurred on the propyl ester as the sole source of carbon. As the colony size increased, crystals of propylparaben were dissolved in the area adjacent to the growth, presumably by the activity of extracellular diffusible enzymes elaborated by the organism (Fig. 1). In contrast, growth did not occur when methylparaben and propyl-

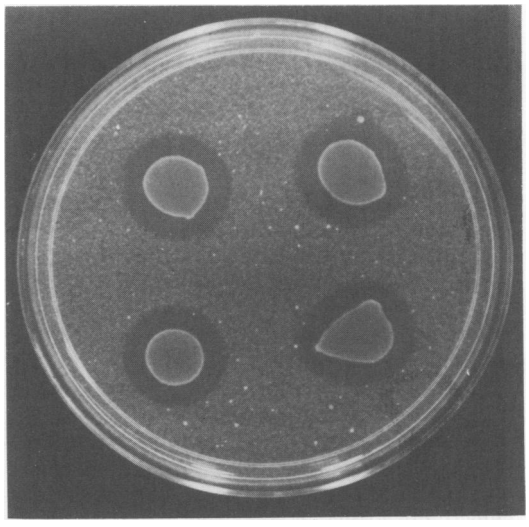

FIG. 1. *Growth of emulsion isolate on paraben media. Utilization of crystals of propylparaben is demonstrated by the clear zones surrounding growth. Growth did not occur on media containing: 0.1% methylparaben; 0.1% methylparaben and 0.1% propylparaben; 0.1% methylparaben and 0.1% glucose.*

paraben were both present in the medium, when methylparaben was substituted for propylparaben, or when 0.1% glucose was added to methylparaben.

Growth patterns of strain 2487 in experimental formulations. Eugon agar-grown cells introduced into emulsions containing methylparaben, either alone or in combination with propylparaben, were reduced to less than 10 organisms/g within day 1. Resistant survivors, however, proliferated and by day 6 had reached the level observed in the unpreserved control emulsion (Fig. 2A and C).

The formulation containing propylparaben alone was ineffective in inhibiting growth of both Eugon and propylparaben agar-derived inocula (compare Fig. 2B and D), indicating resistance of strain 2487 to the inhibitory action of this compound. This was anticipated inasmuch as earlier observations had shown that the organism was able to utilize the propyl ester as the sole source of carbon and energy.

Cells grown on propylparaben agar showed an initial drop in viability when inoculated into emulsions containing methylparaben or methylparaben plus propylparaben. However, by day 3 the number of cells had returned to the original level. This suggests that either the propylparaben-resistant population contained a few methylparaben-resistant cells or that the esterase present in the propylparaben-derived inoculum was not specific for the propyl side chain, as suggested by Ornston (8), and that after a short induction period the organism was capable of hydrolyzing the methyl ester as well.

Chromatographic assay of paraben content. To confirm the ability of *P. cepacia* to attack paraben esters, the amount of parabens remaining after cellular growth in emulsions was determined by chromatographic assay. The percent decrease in total paraben content of emulsions was calculated for each sample (Table 1). The values obtained indicate that methylparaben was selectively destroyed when both esters were present and that methylparaben was degraded to a greater extent by cells grown on propylparaben agar.

The greatest decrease observed was that of methylparaben in the emulsion containing methylparaben only and inoculated with propylparaben agar-grown cells. Furthermore, when both esters were present, the methyl was selectively attacked, irrespective of the origin of the inoculum. This was somewhat unexpected since strain 2487 was unable to utilize the methyl ester as a substrate for growth in minimal media.

The results show decreases in the concentration of propylparaben in the propylparaben-containing emulsions inoculated with both Eugon and propylparaben agar-grown cells. Less propylparaben remained in the sample inoculated with propylparaben agar-grown cells, probably due to a higher concentration of propylparaben esterase elaborated by the cells. In addition to the amounts of the esters calculated to be present by analysis of chromatographic data, a consistent appearance of *p*-hydroxybenzoic acid was also observed in chromatographic traces whenever the concentration of methylparaben or propylparaben decreased, although not in stoichiometric proportions. Peaks for *p*-hydroxybenzoic acid were absent in the uninoculated controls.

DISCUSSION

Esters of *p*-hydroxybenzoic acid are frequently used in combination to increase preservative effectiveness by broadening the antimicrobial spectrum and increasing their concentration in solution (3). This work demonstrated that a strain of *P. cepacia* isolated from a paraben-preserved emulsion is capable of destroying both the methyl and propyl esters of *p*-hydroxybenzoic acid and can, additionally, utilize propylparaben as the sole source of carbon and energy in minimal media.

Preferential hydrolysis of methylparaben in the experimental emulsions inoculated with cells grown on propylparaben agar may reflect the specificity of a single paraben esterase elaborated by strain 2487.

Alternatively, if there is more than one enzyme, methylparaben may repress the propylparaben esterase. This could explain the growth inhibition observed with the methyl and propyl esters in combination (Fig. 2C), even though the enzymes can hydrolyze both esters separately. Another explanation depends upon the recognition that the propylparaben partitions preferentially into the oil phase and is not as available to the organism, except at the oil-water interfaces. This understanding of the ester hydrolysis pattern in emulsion systems depends upon the fact that propylparaben partitions better into the oil phase than does the methyl homologue and that the ratio of concentrations in the aqueous phase is very different from that ratio in the entire emulsion. None of these explanations contradicts the fact that propylparaben is hydrolyzed in aqueous systems, since this compound can be utilized as a sole carbon source. This would also help explain the ability of the organism to attack propylparaben in an aqueous agar medium. Diffu-

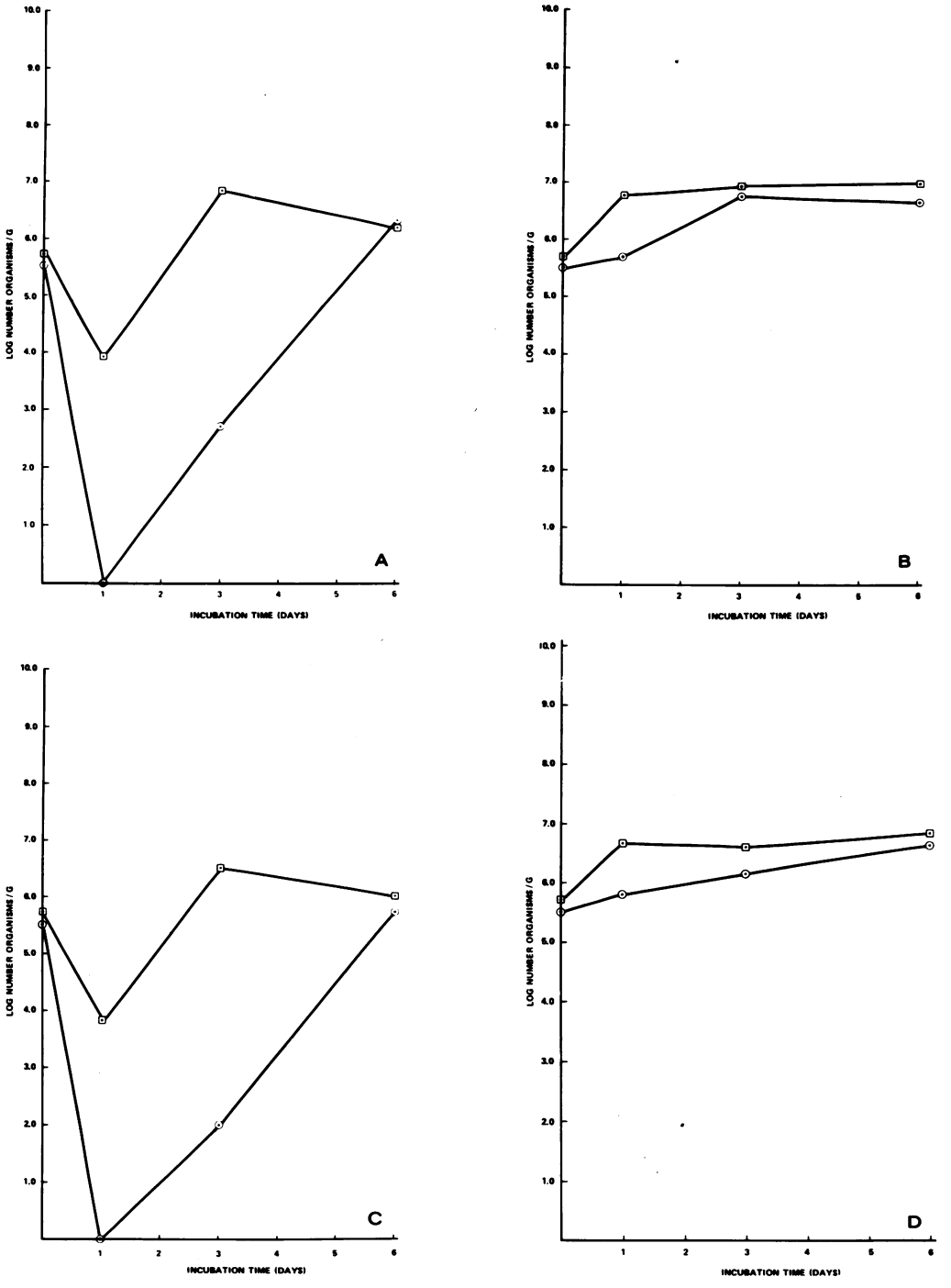

FIG. 2. *Growth of P. cepacia in cosmetic emulsions observed for 6 days. (A) Preserved emulsion—methylparaben; (B) preserved emulsion—propylparaben; (C) preserved emulsion—methylparaben plus propylparaben; (D) unpreserved emulsion control. The samples were inoculated with cells prepared from 24-h cultures grown on Eugon agar (⊙) or propylparaben agar (⊡).*

TABLE 1. *Paraben levels after storage of inoculated emulsion samples for 1 month at 25 C*

Preservative in sample emulsion	Eugon agar-grown cells		Propylparaben agar-grown cells		Uninoculated control	
	Parabens conc (g/100 g)	Decrease (%)	Parabens conc (g/100 g)	Decrease (%)	Parabens conc (g/100 g)	Decrease (%)
Methylparaben	0.164	3.52	0.050	70.5	0.170	0
Propylparaben	0.076	24.0	0.052	48.0	0.100	0
Methylparaben plus Propylparaben	0.159 0.101	6.47 0	0.123 0.101	27.6 0	0.170 0.1	0 0

sible enzymes would dissolve the crystalline material as needed, whereas in the emulsion propylparaben is dissolved in a water-immiscible solvent (6, 12).

The exact mechanism by which strain 2487 breaks down and utilizes the esters of *p*-hydroxybenzoic acid was not determined. The first step is quite likely by means of esterases, as described by Stanier et al. (11). The ability of this organism to utilize *p*-hydroxybenzoic acid esters, despite the initial inhibition of cells in the presence of methylparaben, is probably due to the partially inducible nature of the enzyme for methyl ester breakdown (8). Sokolski et al. (10) demonstrated an initial suppression of growth with subsequent resistance to methyl *p*-hydroxybenzoic acid by *Cladosporium resinae*. Beveridge and Hart (2) measured the degree of utilization of several *p*-hydroxybenzoate esters by various *Pseudomonas* species.

Although *p*-hydroxybenzoic acid appears in emulsion samples as the concentration of the esters decreases and is related to cellular growth, it does not appear to occur in amounts corresponding to loss of the esters. Thus, the possible further breakdown of *p*-hydroxybenzoic acid to other substances, which may in turn be metabolized, remains a subject for study.

Routine challenge testing of experimental formulations can be expanded when necessary to include nutritionally versatile organisms, as represented here by *P. cepacia*, in addition to the usual USP type strain challenges. Observance of good manufacturing procedures, including monitoring of raw materials to avoid contamination and the resulting reduction of preservative capacity, is a prudent preventive measure.

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