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Metabolism of Pyridine Compounds by Phthalate-Degrading Bacteria

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Bacteria were isolated from marine sediments that grew aerobically on *m*-phthalate, *p*-phthalate, or dipicolinate (2,6-pyridine dicarboxylate [2,6-PDCA]). Strain OP-1, which grew on *o*-phthalate and was previously obtained from a marine source, was also studied. Intact cells of each organism demonstrated Na^+-dependent oxidation of their growth substrates. Strain PCC5M grew on dipicolinate but did not metabolize *m*-phthalate. The phthalate degraders, however, demonstrated Na^+-dependent metabolism of the appropriate PDCA analogs. 2,6-PDCA was transformed by strain CC9M when this strain was grown on *m*-phthalate, 2,5-PDCA was metabolized by strain PP-1 grown on *p*-phthalate, and 2,3-PDCA (quinolinate) was oxidized by strain OP-1 grown on *o*-phthalate. Spectral changes accompanying the Na^+-dependent transformations of the PDCA analogs suggest the formation of hydroxylated compounds. Metabolism probably occurred via phthalate hydroxylases; this is a previously unrecognized route for the environmental transformation of pyridine compounds. Hydroxylated products may feed into known pathways for the catabolism of pyridines or be photochemically degraded because of their absorbance in the solar actinic range (wavelengths > 300 nm). The results reinforce recent evidence for the broad potential of aromatic hydroxylase systems for the destruction of pollutants.

Phthalate esters are industrial chemicals which are produced in vast quantities, mainly because of their use as plasticizers (4, 10). The presence of phthalate esters in natural materials is probably the result of contamination with synthetic compounds (18). Microorganisms have been readily isolated, from a variety of environments, which degrade phthalic acids and their esters even though they are xenobiotic compounds (6, 12). The possibility that bacteria growing on quinolinate (2,3-pyridine dicarboxylate [2,3-PDCA]), a naturally occurring structural analog of *o*-phthalate, might contribute to the degradation of *o*-phthalate was recently considered (17). A mixed culture that grew on 2,3-PDCA did not metabolize *o*-phthalate. In contrast, strain OP-1, a marine bacterium that grew aerobically on *o*-phthalate (15), metabolized 2,3-PDCA when grown on *o*-phthalate. The current report describes studies on the possible interrelationships in the bacterial metabolism of *m*-phthalate (isophthalate) and *p*-phthalate (terephthalate) and their pyridine analogs. Pure cultures of marine isolates grown on *m*- or *p*-phthalate partially metabolized the appropriate structural PDCA, but a marine bacterium that grew on 2,6-PDCA (dipicolinate), a component of bacterial spores (14), did not metabolize *m*-phthalate. Although pyridine degraders do not appear to promote the biodegradation of phthalates, it is likely that enzymes involved in the hydroxylation of aromatic compounds contribute to the dissimilation of pyridine derivatives.

MATERIALS AND METHODS

Media and cultures. A synthetic medium with the major salts at about one-half the strength of those in seawater was used: NaCl, 13.7 g; $MgSO_4 \cdot 7H_2O$, 3.45 g; $MgCl_2 \cdot 6H_2O$, 2.6 g; $CaCl_2 \cdot 2H_2O$, 0.735 g; KCl, 0.165 g; NH_4Cl, 0.3 g; KH_2PO_4, 0.05 g; yeast extract, 0.25 g; SL4 trace metals (11), 10 ml; Tris hydrochloride, 0.79 g; MilliQ water, 1 liter. The pH was adjusted to 8.0 before the medium was autoclaved. Growth substrates were added in 5 mM increments. Strain OP-1 was previously isolated from a marine mud (16); it is a marine bacterium that requires Na^+ for aerobic growth on, and metabolism of, *o*-phthalate (15). The following organisms were isolated from marine sediments by aerobic elective culture: strain CC9M, on *m*-phthalate; strain PP-1, on *p*-phthalate; and strain PCC5M, on 2,6-PDCA. Cells were grown in darkness in 100-ml batches in 500-ml Erlenmeyer flasks at 30°C with rotary shaking (200 rpm). Growth was assessed with a Klett-Summerson colorimeter. Cells were harvested by centrifugation and washed in 0.05 M Tris buffer (pH 8.0) containing 0.2 M NaCl, 0.05 M $MgSO_4$, and 0.01 M KCl. In Na^+-free experiments, the 0.2 M NaCl was omitted.

Cell suspension experiments. Oxygen uptake was measured at 30°C with a Clark-type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). The reaction vessel contained 5 ml of cell suspension. The endogenous respiratory rate was recorded, and then the substrate was added to yield a final concentration of 20 μM. After 10 to 15 min, samples were removed and centrifuged to remove cells before spectrophotometric analysis of the supernatant solutions. The supernatants were diluted with an equal volume of water, and the spectra were measured against a water blank in 1-cm-light-path cuvettes with a model 8450A spectrophotometer (Hewlett-Packard Co., Palo Alto, Calif.). Protein was determined by the Biuret method (5).

Materials. Chemicals and biochemicals were bought from either Aldrich Chemical Co., Inc. (Milwaukee, Wis.) or Sigma Chemical Co. (St. Louis, Mo.). Agar and yeast extract were bought from Difco Laboratories (Detroit, Mich.).

RESULTS

Cells of strain PCC5M grown on 2,6-PDCA exhibited an Na^+-dependent oxidation of 2,6-PDCA but did not oxidize *m*-phthalate, even in the presence of Na^+ (Table 1). The rate of 2,6-PDCA oxidation was not affected by equimolar levels of *m*-phthalate. Cells which had been maintained for 2 to 3 h

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TABLE 1. Oxidation of 2,6-PDCA and *m*-phthalate by strain PCC5M grown on 2,6-PDCA

Substrate	Na^+ added	Oxygen uptake (nmol/min per mg of protein)		
		Plus substrate	Endogenous	Net
2,6-PDCA	+	26.0	7.0	19.0
	+[a]	25.2	6.8	18.4
	−	3.6	3.6	0
m-Phthalate	+	6.8	6.8	0
2,6-PDCA + *m*-phthalate	+	23.5	6.8	16.7

[a] 160 mM NaCl added back to cells after 2 to 3 h in the absence of Na^+.

without Na^+ immediately demonstrated activity for 2,6-PDCA oxidation upon addition of Na^+ (Table 1). Cells of the phthalate-degrading bacteria oxidized both phthalates and PDCAs (Table 2). Stimulation of oxygen uptake rates by PDCAs was limited to those PDCAs bearing structural relationships to the phthalate substrate. Strain OP-1 (*o*-phthalate grown) oxidized 2,3-PDCA but not 2,5-PDCA or 2,6-PDCA, strain CC9M (*m*-phthalate grown) oxidized 2,6-PDCA but not 2,3-PDCA or 2,5-PDCA, and strain PP-1 (*p*-phthalate grown) used 2,5-PDCA but not 2,3-PDCA or 2,6-PDCA.

Both strain CC9M and strain PP-1 showed Na^+-dependent oxidation of phthalates and their PDCA analogs (Fig. 1). Changes in the spectra of the PDCAs occurred upon incubation with cells grown on phthalates but only when Na^+ was present (Fig. 2). As with 2,6-PDCA, cells incubated without Na^+ for about 2 to 3 h fully retained their ability to oxidize *m*-phthalate (strain CC9M) and *p*-phthalate (strain PP-1) upon addition of Na^+. The product(s) of 2,6-PDCA metabolism had new absorption maxima at about 308, 319, and 333 nm, whereas the products of 2,5-PDCA metabolism had new bands at about 302 and 332 nm. The spectra of the products from all three PDCAs showed pH-dependent changes with, in general, shifts to longer wavelengths for absorbance maxima or shoulders as the pH was increased (Table 3). A similar shift was seen with 2,3-pyridinediol but not 6-hydroxynicotinate (Table 3).

DISCUSSION

Our results extend previous studies on the metabolism of 2,3-PDCA by strain OP-1 grown on *o*-phthalate (17). The structural relationship between the PDCA and the phthalate isomer that were oxidized indicates metabolism of PDCAs via enzymes for phthalate catabolism. Na^+-dependent stimulation of oxygen uptake and Na^+-dependent spectral changes, as in the metabolism of phthalates, indicate uptake of the PDCAs via phthalate permeases. Na^+-dependent metabolism is common in marine bacteria and usually reflects the participation of Na^+ in the mechanisms for the transport of nutrients through the cytoplasmic membrane and into the cytoplasm (7).

TABLE 2. Oxidation of PDCAs and phthalates by cells grown on phthalates

Strain	Oxygen uptake (nmol/min per mg of protein)[a]			
	Phthalate[b]	2,3-PDCA	2,5-PDCA	2,6-PDCA
OP-1	161	10	0	0
CC9M	265	0	0	24
PP-1	292	0	32	0

[a] Endogenous rates subtracted.
[b] *o*-Phthalate for strain OP-1, *m*-phthalate for strain CC9M, and *p*-phthalate for strain PP-1.

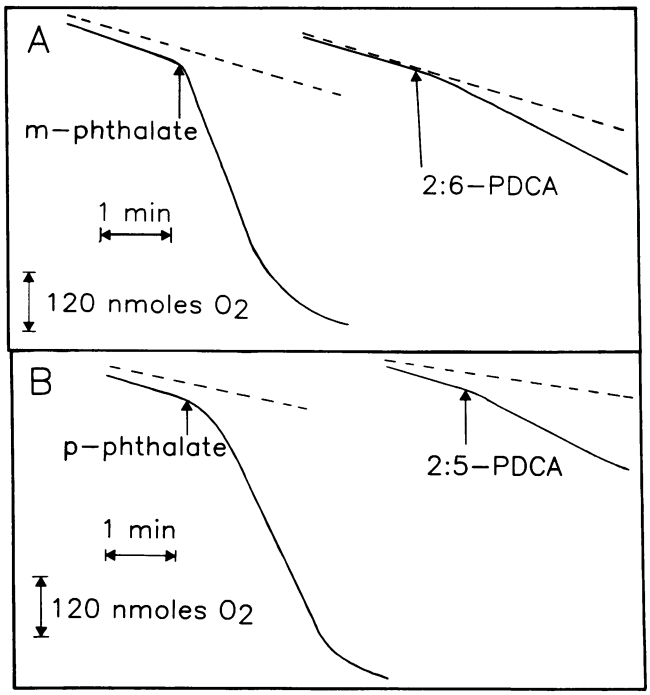

FIG. 1. Oxidation of *m*-phthalate and 2,6-PDCA by strain CC9M grown on *m*-phthalate (A) and of *p*-phthalate and 2,5-PDCA by strain PP-1 grown on *p*-phthalate (B). Substrates were added at the times indicated by arrows. ----, Na^+ absent; ——, Na^+ present.

Increased absorbance in the 300- to 350-nm region resulting from the metabolism of PDCAs is probably caused by the introduction of hydroxyl groups onto the heterocyclic ring (17). The spectra of the metabolites from all three PDCAs showed pH-dependent changes with, in general, shifts to longer wavelengths for absorbance maxima or shoulders as the pH increased (Table 3). A similar spectral shift occurred with 2,3-pyridinediol but not with 6-hydroxynicotinate, which has only one hydroxyl group (Table 3). The pH-dependent changes may indicate the formation of *o*-dihydroxypyridines since pyridones with vicinal hydroxyl groups show bathochromic shifts in alkaline solutions, whereas those with *m*-hydroxyls display hypsochromic shifts (1). Persistence of absorbance in the 260-nm region suggests that PDCA metabolism does not involve ring cleavage. Aromatic ring fission enzymes may not use dihydroxypyridines because of their existence as hydroxypyridones at neutral pH due to ketonization of 2- and 4-hydroxyl substituents (8).

If aromatic ring cleavage dioxygenases do not accept the products from the phthalate hydroxylases, other mechanisms must be considered for further degradation of the PDCA metabolites. Hydroxylated pyridines may enter aerobic or anaerobic catabolic pathways (2, 13, 20) or, because of their absorbance at wavelengths longer than those of the parent PDCAs, be susceptible to photochemical destruction. The product of 2,3-PDCA metabolism, for example, is far more photolabile than 2,3-PDCA itself (unpublished data).

Recent studies have drawn attention to the broad specificities of some aromatic-compound-hydroxylating systems, especially toluene dioxygenase and its diol dehydrogenase.

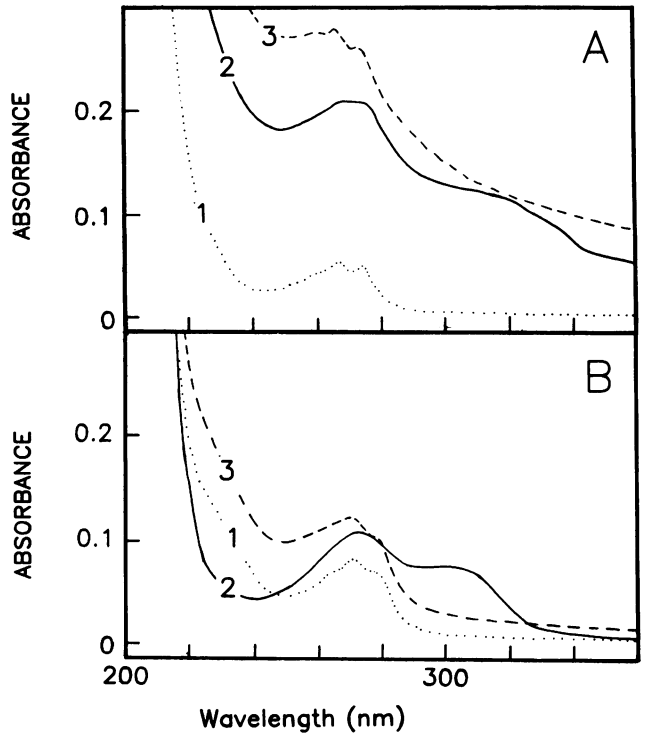

FIG. 2. Spectral changes accompanying the metabolism of 2,6-PDCA by strain CC9M grown on *m*-phthalate (A) and of 2,5-PDCA by strain PP-1 grown on *p*-phthalate (B). 1, Initial spectrum; 2, spectrum after incubation, Na^+ present; 3, spectrum after incubation, Na^+ absent.

This hydroxylating system, which accepts a wide variety of aromatic hydrocarbons (3), also attacks trichloroethylene (9) and even functions as a monooxygenase with some substrates (19). The toluene-hydroxylating enzymes and other aromatic-compound-hydroxylating complexes may also accept pyridine analogs. Aromatic and heterocyclic compounds often occur together in environments, either naturally or as pollutants, and so cometabolism would be feasible even if the pyridine analogs are not inducers of the aromatic enzymes. The suites of enzymes responsible for the initial metabolic steps for each phthalate isomer probably have relaxed specificities. Phthalate hydroxylases deserve investigation for their potential use in the destruction of aromatic, heterocyclic, and even aliphatic pollutants.

TABLE 3. Effect of pH on the spectra of PDCA metabolites, 2,3-pyridinediol and 6-hydroxynicotinate

Compound	Absorbance bands (nm)[a] at:		
	pH 1	pH 8	pH 13
2,3-PDCA metabolite	273 (0.229)	269 (0.254)	269 (0.196)
	314 (0.108)	308 (0.189)	317 (0.095)
		319 (0.202)	328 (0.103)
		333 (0.137)	342 (0.076)
2,6-PDCA metabolite	260 (0.173)	271 (0.156)	279 (0.123)
	308 (0.171)	308 (0.200)	320 (0.118)
	319 (0.158)	320 (0.220)	330 (0.131)
	334 (0.096)	333 (0.148)	342 (0.096)
2,5-PDCA metabolite	250 (0.118)	273 (0.233)	269 (0.238)
	292 (0.142)	302 (0.180)	300 (0.156)
		332 (0.028)	343 (0.030)
2,3-Pyridinediol	230 (0.103)	248 (0.124)	254 (0.113)
	293 (0.138)	304 (0.166)	309 (0.109)
6-Hydroxynicotinate	258 (0.201)	250 (0.190)	262 (0.215)
	293 (0.073)	296 (0.082)	293 (0.105)

[a] Values in parentheses are absorbances.

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