Isolation and Characterization of a Chlorinated-Pyridinol-Degrading Bacterium

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The isolation of a pure culture of bacteria able to use 3,5,6-trichloro-2-pyridinol (TCP) as a sole source of carbon and energy under aerobic conditions was achieved for the first time. The bacterium was identified as a *Pseudomonas* sp. and designated ATCC 700113. [2,6-¹⁴C]TCP degradation yielded ¹⁴CO₂, chloride, and unidentified polar metabolites.

3,5,6-Trichloro-2-pyridinol (TCP) has been detected as a major metabolite in environments where the herbicides chlorpyrifos and triclopyr had been applied (3, 6, 10, 12, 15, 21). TCP is also present in raw wastewater originating from a chlorpyrifos-producing facility. The mineralization of TCP in soil is microbially mediated, but isolation of the degradative microorganisms has never been reported. Although relatively nontoxic to mammals, TCP exhibits low-to-moderate toxicity to aquatic and terrestrial biota (14). In addition, TCP has displayed some potential to affect microorganisms; soil concentrations higher than 100 ppm have been reported to retard the microbial degradation of several insecticides (2, 15, 16). The objective of this study was to isolate and characterize a pure culture of bacteria capable of using TCP as a sole source of carbon and energy.

Enrichment culture techniques were used to obtain TCP-degrading bacteria from an agricultural soil which had been treated with chlorpyrifos. The mineral salt medium used was described previously (5). When it was necessary to obtain large amounts of cells, 0.01% (wt/vol) yeast extract and 0.018% (wt/vol) glucose were added.

Phenotypic characterization of the isolate was carried out according to methods described by Smibert and Krieg (20). Identification was based on data in *Bergey's Manual of Systematic Bacteriology* (9), the results of Biolog analysis (Biolog, Inc., Hayward, Calif.), and fatty acid analysis (performed by Microcheck, Inc., Northfield, Vt.).

The disappearance of TCP was monitored by high-performance liquid chromatography with detection by UV absorbance at 320 nm as previously described (5). Radioactivity was determined with a Beta Trac 6895 liquid scintillation counter (Tracor Analytic, Elk Grove Village, Ill.). Concentrations of chloride were determined with a chloridometer (Buchler Instruments, Inc., Fort Lee, N.J.).

The TCP-degrading isolate was a gram-negative rod, and colonies on agar plates were circular with entire margins and smooth surfaces. Colonies appeared yellow on nutrient agar and yeast extract-glucose agar, but a brown pigment was produced on tryptic soy and King's B agars. The pigment was nonfluorescent and diffusible on King's B agar plates. The

isolate was catalase positive, cytochrome oxidase positive, and arginine dihydrolase negative. It reduced nitrate to nitrite, used glucose oxidatively, and hydrolyzed gelatin but not starch. It used L-arabinose, D-arabitol, D-fructose, D-galactose, α -D-glucose, inositol, D-mannitol, D-mannose, D-sorbitol, sucrose, and D-trehalose but not N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, cellobiose, erythritol, L-fructose, gentiobiose, α -D-lactose, lactulose, maltose, β -methyl-D-glucoside, D-raffinose, L-rhamnose, turanose, and xylitol. Cells grown on TCP did not cleave the aromatic ring of catechol at either the *ortho* or the *meta* position by methods described by Smibert and Krieg (20).

The hypersensitive reaction and inoculation of tomato stems were carried out to detect the pathogenicity of the isolated bacteria with respect to plants (6, 11). Some browning of vascular tissue (1.5- to 2-cm long) was present 6 weeks after stem inoculation of tomato plants, but the plants appeared healthy. The bacteria were reisolated from the plants. The isolate did not result in a positive hypersensitive reaction on tobacco leaves.

While the results from Biolog's identification system indicated with a probability of 60% that the isolate was *Pseudomonas corrugata*, the fatty acid profile of the isolate matched that of *Pseudomonas marginalis* with a similarity index of 0.867. The phenotypic features of the isolate agreed with the general descriptions for *Pseudomonas* species but did not completely agree with those reported for either organism in the literature (8, 9). The isolate displayed many characteristics of *P. corrugata*; however, its fatty acid profile did not match that of *P. corrugata* and it did not cause tomato pith necrosis or the incompatible hypersensitive response on tobacco leaves. The isolate differed from *P. marginalis* in that the latter generates different "breathprints" on a Biolog microplate, produces fluorescent pigments on King's B medium, and grows under denitrifying conditions. Thus, the isolate was designated a *Pseudomonas* sp., deposited in the general collection of the American Type Culture Collection, and assigned the accession number ATCC 700113. In this report, the bacterium is referred to as *Pseudomonas* sp. strain ATCC 700113 (previously called strain M285) (4).

Pseudomonas sp. strain ATCC 700113 grown in a mineral salt medium (5) was able to mineralize [2,6-¹⁴C]TCP readily (Fig. 1). About 72.4% of the initial radioactivity was recovered as ¹⁴CO₂, while 9% remained in the medium and 4.1% remained in the biomass; the remainder may be accounted for by losses in the form of ¹⁴CO₂ during the sampling process. Chloride was released stoichiometrically and concurrently with the

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FIG. 1. Mineralization of TCP by *Pseudomonas* sp. strain ATCC 700113. The bacteria were grown at 28°C in mineral salt medium.

disappearance of TCP. Theoretically, complete mineralization of 40 mg of TCP/liter of medium should release 21.6 mg of chloride ion per liter. A chloride concentration of 25.7 mg/liter was detected in the culture after the disappearance of TCP. Accumulation of ammonium in the ammonium-free medium was detected qualitatively with Nessler's reagent.

Manipulation of experimental conditions revealed that TCP was most rapidly degraded at a temperature of 28°C and a pH between 6.2 and 7.8. When the effects of various TCP concentrations were studied, the degradation of TCP was complete within 3 weeks at concentrations of 100 mg/liter or less but degradation was not observed at 200 mg/liter. Transformation of TCP was also affected by the presence of NaCl; transformation decreased with increasing concentrations of NaCl and was completely inhibited at 10 g/liter. Degradation was also slowed down by the addition of 40, 75, and 150 mg of chloramphenicol/liter but was stimulated by the addition of carbon sources such as glucose, maleic acid, and succinic acid. Ammonium ion at 3.8 mM (68 mg/liter) did not affect TCP degradation, but a concentration of 15 mM (270 mg/liter) was slightly inhibitory. The TCP-degrading ability of *Pseudomonas* sp. strain ATCC 700113 was a stable trait, since the organism degraded TCP after repeated subcultures on TCP-free medium.

For the preparation of resting cells, *Pseudomonas* sp. strain ATCC 700113 was grown in the mineral salt medium containing 100 mg of TCP/liter, 0.01% yeast extract, and 0.018% glucose. Cells were harvested, washed three times, and resuspended in 50 mM phosphate buffer to an optical density of 1.0

FIG. 2. Metabolism of TCP by resting cells of *Pseudomonas* sp. strain ATCC 700113. Cells were suspended in 50 mM phosphate buffer (optical density at 630 nm = 1.0) containing 40 mg of TCP/liter and incubated at 28°C.

(172 µg of protein/ml). TCP (40 mg/liter) was completely degraded in 70 h by these resting cells (Fig. 2). About 87% of the initial radioactivity was recovered as ¹⁴CO₂, 3% remained in the medium, and 1.8% was associated with the biomass. The high-performance liquid chromatography chromatogram revealed the presence of some polar metabolites; however, the amounts were insufficient for further characterization. Resting cells of *Pseudomonas* sp. strain ATCC 700113 did not metabolize 2-, 3-, and 4-hydroxypyridines, 2,4,5-trichlorophenol, and 2,4,5-trichloroaniline.

Crude cell extracts of *Pseudomonas* sp. strain ATCC 700113 were prepared by grinding cells with powdered alumina with a mortar and pestle (7). Cell extracts were incubated with 40 mg of TCP/liter in 20 mM phosphate buffer supplemented with various cofactors. Cell extracts did not degrade TCP regardless of additions of NAD⁺, NADH, flavin adenine dinucleotide, and ferrous ion. Earlier studies of pyridine also failed to obtain pyridine-degrading cell extracts (1, 19).

Pseudomonas sp. strain ATCC 700113 was able to mineralize [2,6-¹⁴C]TCP, as was demonstrated by the release of ¹⁴CO₂ and chloride ions. However, only 4.1% of the initial radioactivity was incorporated into the biomass of growing cell cultures and 1.8% was incorporated in resting cell cultures. The cells appeared to be carbon starved while they were grown on TCP alone, since addition of a second carbon source stimulated the growth of *Pseudomonas* sp. strain ATCC 700113 and, as a result, increased the rate of TCP transformation. Low ¹⁴C distribution in biomass has also been reported for bacteria grown on [U-¹⁴C-ring]atrazine (<3%) (18) and [N-¹⁴C]methylisonicotinate (<6%) (13).

Although mineralization of TCP in soil has been reported (15, 17), this is the first report on the isolation and characterization of a pure culture of bacteria capable of mineralizing TCP and using it as a sole source of carbon and energy.

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