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Improved Degradation of Monochlorophenols by a Constructed Strain

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Pseudomonas sp. strain B13, a strain able to degrade 3-chlorobenzoate and, after prolonged adaptation (40 days), 4-chlorophenol, could transfer the ability to degrade chlorocatechols to a recipient, *Alcaligenes* sp. strain A7, which is able to grow with benzoate and phenol. Representative transconjugants, such as *Alcaligenes* sp. strain A7-2, were able to utilize all three isomeric chlorophenols; this property was not possessed by the donor or the recipient. The ability to grow readily with 4-chlorophenol may be attributable to a more rapid induction of phenol hydroxylase by *Alcaligenes* sp. strain A7-2 than by *Pseudomonas* sp. strain B13, a property which correlates with the greater level of resistance to chlorophenols shown by the transconjugant.

Few bacteria which can utilize chlorinated phenols as the sole source of carbon and energy have been described. Tyler and Finn (14) described a 2,4-dichlorophenoxyacetate-degrading pseudomonad which also utilizes 2,4-dichlorophenol. A 3-chlorobenzoate-degrading strain, *Pseudomonas* sp. strain B13, which is able to utilize 4-chlorophenol as the sole source of carbon and energy, has been isolated (4, 9). This organism can cooxidize 2- and 3-chlorophenol but cannot utilize these xenobiotics for growth.

The ability of *Pseudomonas* sp. strain B13 to utilize 3-chlorobenzoate or 4-chlorophenol is essentially based on the induction of at least three enzymes which, in contrast to ordinary enzymes of the 3-oxoadipate pathway, exhibit unusual substrate specificities. The isoenzymes pyrocatechase II and cycloisomerase II are distinguished by high activity for chlorinated substrates (6). The hydrolysis of 4-carboxymethylenebut-2-en-4-olide generated by dehalogenating cycloisomerization of chloromuconic acids is catalyzed by a third enzyme, which functions exclusively in the chloroaromatic pathway (12).

This paper describes an *Alcaligenes* strain which can tolerate high concentrations of phenol and which, by transfer of the halocatechol-degrading capacity from *Pseudomonas* sp. strain B13, acquires the ability to utilize all three isomeric chlorophenols without long adaptation procedures.

MATERIALS AND METHODS

Organisms. *Pseudomonas* sp. strain B13 has been described by Dorn et al. (4). *Alcaligenes* sp. strain A7 was isolated from a continuous culture which was inoculated with soil samples and which degraded certain components of synthetic sewage. This culture was screened for organisms that could degrade phenol at high concentrations in addition to other components of industrial sewage such as ethanol, methanol, isopropanol, and acetone.

Alcaligenes sp. strain A7-2 was isolated during a conjugation experiment described below.

Media and culture conditions. For growth in liquid culture, the mineral medium described by Dorn et al. (4) containing benzoate, 3-chlorobenzoate, or chlorophenols was used. To avoid acidification during growth on chlorinated substrates, the buffer concentration was increased twofold; this did not affect the growth rate. Cells were grown in batch cultures with benzoate or 3-chlorobenzoate as growth substrates in fluted Erlenmeyer flasks incubated at 28°C on a rotary shaker at 150 rpm. Continuous cultures with chlorophenols as substrates were grown at 30°C in 250-ml chemostats as described by Knackmuss and Hellwig (9) or in a 2-liter fermentor (Biolafitte, Poissy, France) containing 1 liter of medium. Fresh medium was added continuously by means of a peristaltic pump (LKB, Bromma, Sweden). Polyethylene glycol at a final concentration of 0.003% (vol/vol) was added to the medium as an antifoaming agent.

Solid medium was prepared by the addition of agar (Difco Laboratories, Detroit, Mich.) to solutions of the basal medium. Kanamycin-resistant strains were grown on solid medium containing 35 μg of kanamycin per ml.

To follow the growth of cell suspensions in batch cultures, 20 ml of these cultures was incubated in fluted 100-ml sidearm flasks, in which turbidity was measured with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.; filter no. 54). An absorbance at 546 nm of 0.63 corresponded to about 100 Klett units. The flasks were sealed with rubber stoppers and agitated in a rotary water bath shaker.

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Taxonomic investigations. Several tests were performed with the API system (API S.A., La Balme Les Grottes, France). The API 20E gallery is a standardized miniaturized version of conventional procedures for the identification of *Enterobacteriaceae* and other bacteria. It is a ready-to-use microtube test system designed for performing numerous biochemical tests. The tests used here are summarized below.

The procedure described by Doetsch (3) was used for determining the Gram reaction.

A simple method, described by Mayfield and Innis (10), was used for staining bacterial flagella.

Catalase was detected by adding 3% H_2O_2 directly to the growth on the plates. Oxidase was detected with filter paper strips saturated with a 1% aqueous solution of tetramethyl-*p*-phenylenediamine.

Preparation of cell-free extracts. Cell suspension in 20 mM Tris-hydrochloride buffer (pH 8.0) were disrupted by using a French press (Aminco, Silver Spring, Md.). Cell debris and particles were removed by sedimentation at 100,000 × *g* for 60 min.

Assays for pyrocatechase. Catechol 1,2-dioxygenase (EC 1.13.11.1) activity was measured by the procedure of Dorn and Knackmuss (5). Extinction coefficients for the ring fission products were those reported by Dorn and Knackmuss (6).

Oxygen uptake experiments with whole cells. Cells of *Pseudomonas* sp. strain B13 or *Acaligenes* sp. strain A7-2 were grown in continuous culture with monochlorophenol as the substrate. Further details of substrate concentration and dilution rate are given in Table 2. A 2-ml amount of 66 mM phosphate buffer (pH 7.3) was added to 1 ml of the cell suspension. After vigorous aeration for 8 min, the cell suspension was placed in the reaction vessel of the oxygen analyzer (type Ysi 5350; Yellow Springs Instruments, Yellow Springs, Ohio). At zero time 100 μl of 10 mM phenol or 10 mM chlorophenol was introduced into the reaction chamber by means of a calibrated syringe. At 30°C the oxygen concentration in the air-saturated cell suspension was taken to be 0.26 mM.

The protein content of the cell suspensions was determined according to Schmidt et al. (13).

Analytical methods. The concentrations of chlorinated phenols were determined by high-pressure liquid chromatography by using a reverse-phase Mikro-Pak-C-H column (Varian, Palo Alto, Calif.). Vacuum-degassed 10 mM phosphoric acid containing 0.5% (vol/vol) propanol and 56% (vol/vol) methanol was used as the eluent. Peaks were detected in a variable-wavelength model 635 spectrometric detector (Varian-Techtron, Melbourne, Victoria, Australia) and were identified with authentic samples by their retention times.

RESULTS

Characterization of *Alcaligenes* sp. strain A7. *Alcaligenes* sp. strain A7 was chosen for this investigation because of its ability to grow on agar plates containing 2 mM phenol. In contrast, *Pseudomonas* sp. strain B13 could not grow at this concentration of phenol.

The cells of *Alcaligenes* sp. strain A7 were gram negative and were motile by peritrichously inserted flagella. The number of flagella varied between three and eight. During exponential growth, cells exhibited a length of 3 to 4 μm and a diameter of 1.5 μm. In the stationary phase, cells were smaller and almost coccoid and aggregated in rows of up to 10 cells. Growth was observed even at pH 5.6. When grown on nutrient broth, the organism showed the ability to accumulate poly-β-hydroxybutyric acid. Tests for oxidase and catalase gave positive results. On nutrient broth agar, yellow pigments were formed. Acid was produced under aerobic conditions from fructose or ethanol. Cells exhibited resistance to streptomycin up to about 300 μg/ml of medium and to kanamycin up to 50 μg/ml of medium.

Good growth was observed on the following carbon sources: 2-oxogluconate, sebacate, acetate, citrate, succinate, glucose, salicylate, nicotinate, benzoate, phenol, acetone, isopropanol, ethanol, and autotrophic medium (H_2-CO_2-O_2, 80:10:10).

No growth was observed on starch, gelatin, casein, DNA, hexadecane, or methanol or on nutrient broth at 41°C.

The following tests gave negative results: production of acid under anaerobic conditions, gas production from glucose, lecithinase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, production of H_2S, Voges-Proskauer reaction, production of indole, reduction of nitrate, and denitrification. Except for the yellow pigmentation, most other phenotypic characters of the strain coincided with those of *Alcaligenes eutrophus* (7). The pigmentation indicated *Alcaligenes paradoxus* (biotype I), but *A. paradoxus* is not able to grow on nicotinate and possesses only one or two flagella with predominantly subpolar or lateral insertion (1, 2).

Utilization of chloroaromatics. To evaluate the degradative potential of *Alcaligenes* sp. strain A7 for 4-chlorophenol, *Alcaligenes* sp. strain A7 was grown over a period of 3 months in continuous culture (see above) with a mixture of phenol and 4-chlorophenol (5 and 2 mM, respectively). During an additional period of 30 days, the concentration of 4-chlorophenol in the reservoir was increased gradually to 6 mM. Under these conditions, the culture was washed out. This experiment showed that *Alcaligenes* sp. strain A7 could not be adapted to growth on 4-chlorophenol as the sole source of carbon and energy, even after 4 months.

To transfer the genes coding for degradation of chloroaromatics, we performed conjugation experiments between *Pseudomonas* sp. strain B13 and *Alcaligenes* sp. strain A7. Cell suspensions were obtained from exponentially growing cultures on benzoate (*Alcaligenes* sp. strain A7) or 3-chlorobenzoate (*Pseudomonas* sp. strain

B13). Transconjugants were selected on 3-chlorobenzoate–kanamycin agar plates, since neither of the parent strains was able to grow on this medium. From preliminary tests in which the conjugation experiment was performed with diluted suspensions on nutrient broth agar, no transconjugants could be isolated. However, when 0.1 ml of each culture (absorbance at 546 nm = 0.7) was directly mixed on the selective-medium agar plates, brownish colonies appeared on the borders of the plates after 5 to 6 days. The brown color probably arose from the autoxidation of chlorocatechols formed by the parent strain *Alcaligenes* sp. strain A7. Because of a higher concentration of chlorocatechols in the middle of the plates, only transconjugants at the border could survive. When transferred to fresh agar plates, the transconjugants grew without an accumulation of catechols. They were identified as having the background of *Alcaligenes* sp. strain A7 and designated *Alcaligenes* sp. strain A7-2. Strains having the same phenotypic properties as these transconjugants could also be isolated from a continuous mixed culture of *Alcaligenes* sp. strain A7 and *Pseudomonas* sp. strain B13 stressed with 4-chlorophenol.

The ability to degrade 3-chlorobenzoate appeared to be a stable property in *Alcaligenes* sp. strain A7-2. When cultivated in nutrient broth for seven to eight generations, all of 200 colonies tested retained the ability to grow on 3-chlorobenzoate.

The most important difference between *Alcaligenes* sp. strain A7 and *Alcaligenes* sp. strain A7-2 is the ability of the latter to utilize 3-chlorobenzoate or monochlorophenols as the sole source of carbon and energy. Additionally, phenol is degraded via the *meta* pathway by *Alcaligenes* sp. strain A7 and via the *ortho* pathway by *Alcaligenes* sp. strain A7-2.

Type II pyrocatechase as a key enzyme for the breakdown of chloroaromatics. The ability of bacteria to degrade haloaromatics depends mainly on their capacity to cleave chlorocatechols (5). The fast breakdown of these metabolites is essential because of their toxic effects. Therefore, these key activities in the parental strains *Alcaligenes* sp. strain A7 and *Pseudomonas* sp. strain B13 were compared with those in the transconjugant *Alcaligenes* sp. strain A7-2. Even when grown on benzoate, cells of *Alcaligenes* sp. strain A7-2 exhibited higher activities toward chlorinated catechols than did those of *Alcaligenes* sp. strain A7, indicating induction of a type II pyrocatechase (Table 1). When *Alcaligenes* sp. strain A7-2 was cultivated with 3-chlorobenzoate, activities toward chlorocatechols exceeded those of *Pseudomonas* sp. strain B13 grown on the same substrate.

The mixing diagram of Reineke and Knackmuss (11) correlates the relative maximum velocities of 1,2-dioxygenation of catechol and chlorocatechols with the corresponding mixing ratio of the isoenzymes pyrocatechase I and pyrocatechase II from *Pseudomonas* sp. strain B13. The proportion of the relative activities toward the chlorinated catechols in crude extracts of unknown strains, including the activities of two catechol 1,2-dioxygenases, would only fit the diagram if these enzymes exhibited turnover rates similar to those of the pyrocatechases in *Pseudomonas* sp. strain B13. Since the relative turnover rates of the pyrocatechase from *Alcaligenes* sp. strain A7 (Table 1) are similar to those of the pyrocatechase I from *Pseudomonas* sp. strain B13, it seemed permissible to arrange the relative activities of the crude extract of *Alcaligenes* sp. strain A7-2 grown on 3-chlorobenzoate on this diagram. The good correlation of these values in the mixing diagram established the close similarity of the relative turnover rates of the type II pyrocate-

TABLE 1. Specific and relative activities of catechol 1,2-dioxygenases in cell-free extracts of *Alcaligenes* sp. strain A7-2 and its parental strains

Strain	Activity[a] in the following assay substrate:			
	Catechol	3-Chlorocatechol	4-Chlorocatechol	3,5-Dichlorocatechol
Pseudomonas sp. strain B13[b]	780 (100)	113 (14)	158 (20)	94 (12)
Alcaligenes sp. strain A7-2[b]	926 (100)	315 (34)	344 (37)	231 (25)
Alcaligenes sp. strain A7-2[c]	193 (100)	13 (7)	22 (11)	5.6 (3)
Alcaligenes sp. strain A7[c]	155 (100)	<1 (<1)	12 (8)	<1 (<1)

[a] Specific activities are expressed as units per gram of protein. Relative activities given within parentheses are percentages of that for catechol.

[b] The cells were harvested during exponential growth on 3-chlorobenzoate.

[c] The cells were harvested during exponential growth on benzoate.

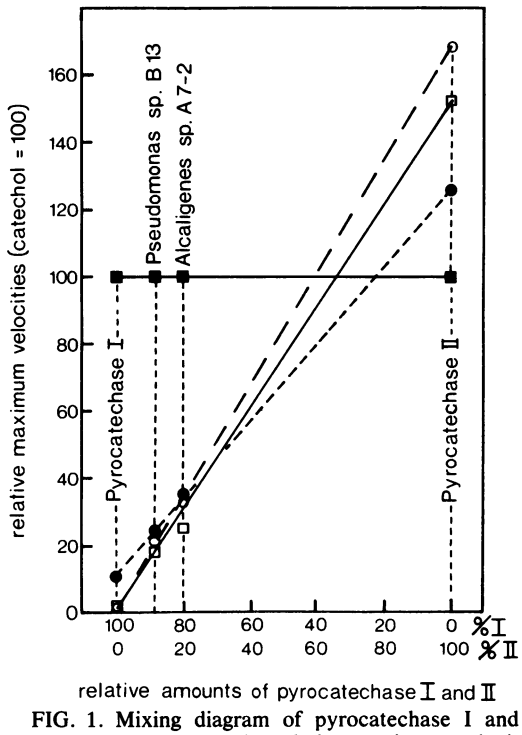

FIG. 1. Mixing diagram of pyrocatechase I and pyrocatechase II (11). The relative maximum velocities of 1,2-dioxygenation of catechol (■) (taken as 100%), 3-chlorocatechol (○), 4-chlorocatechol (●), and 3,5-dichlorocatechol (□) measured with pyrocatechase I from *Pseudomonas* sp. strain B13 are on the left side of the diagram. The data on the right side of the diagram were obtained with pure pyrocatechase II DEAE-cellulose preparations from *Pseudomonas* sp. strain B13. The connecting lines between the data for pyrocatechase I and pyrocatechase II give the theoretical value when both enzymes were mixed. The relative velocities of catechol 1,2-dioxygenation, measured with cell-free extracts of *Pseudomonas* sp. strain B13 and *Alcaligenes* sp. strain A7-2 (Table 1) with different substrates, were entered into the diagram. Both strains were grown on 3-chlorobenzoate.

chase in *Alcaligenes* sp. strain A7-2 to those of the pyrocatechase II of *Pseudomonas* sp. strain B13 (Fig. 1).

Isomeric monochlorophenols as growth substrates. Because of the toxicity of chlorophenols, all strains were maintained on 3-chlorobenzoate agar plates, and batch cultures were cultivated in 3-chlorobenzoate medium. When batch cultures of *Alcaligenes* sp. strain A7-2 or *Pseudomonas* sp. strain B13 growing on 3-chlorobenzoate were supplemented with 4-chlorophenol, both strains exhibited significantly different stabilities. A culture (100 ml) of *Alcaligenes* sp. strain A7-2 growing in batch on 5 mM 3-chlorobenzoate was transferred to a 250-ml chemostat (see above). The cell suspension was supplemented immediately with medium containing 5 mM 4-chlorophenol at a flow rate of 100 ml per day. The culture, which exhibited an absorbance at 546 nm of 0.7, tolerated the change of growth substrates. Even when a medium containing the three isomeric chlorophenols (2 mM each) was admixed, the culture remained stable.

In contrast, under corresponding conditions, a culture of *Pseudomonas* sp. strain B13 could not be adapted to 4-chlorophenol as the growth substrate. A gradual change from 3-chlorobenzoate to 4-chlorophenol over a period of 40 days was required to generate a stable culture of *Pseudomonas* sp. strain B13 utilizing 4-chlorophenol as the sole source of carbon and energy. This culture could not be adapted to the degradation of a mixture of the monochlorophenols. Growth rates of *Alcaligenes* sp. strain A7-2 on a mixture of the monochlorophenols could not be determined in batch culture because of the toxicity of these substrates at high concentrations. Therefore, the doubling rates were estimated by measuring the maximum dilution rate in continuous culture. Cells of *Alcaligenes* sp. strain A7-2 were cultivated in a 2-liter fermentor as described above. The culture was supplemented continuously with medium containing a mixture of the three isomeric monochlorophenols (2 mM each). The flow rate was increased stepwise every 2 days. The culture was washed out when the dilution rate exceeded 3.3 to 4 volume changes per day, corresponding to a doubling time of 6 to 7.2 h.

Inhibition of growth on 3-chlorobenzoate by chlorophenols. To differentiate between the inherent toxicity of the chlorophenol and an inhibitory effect by its metabolites, 3-chlorobenzoate was selected as a growth substrate for the inhibition experiments. This substrate was used because the chlorocatechol-degrading enzymes are already induced, and therefore inhibition by metabolites originating from chlorophenol breakdown could be excluded.

Cultures of *Alcaligenes* sp. strain A7-2 or *Pseudomonas* sp. strain B13 were grown on 3-chlorobenzoate in buffered mineral medium as described above. During exponential growth, both cultures were supplemented with different concentrations of monochlorophenols. The optical density was recorded in Klett units over a period of 4 h. During the entire experiment, 3-chlorobenzoate was present in excess. Control cultures were run without chlorophenols.

The concentrations of the chlorophenols were determined at hourly intervals by high-pressure liquid chromatography. The decrease in the chlorophenols in the medium (Fig. 2) could be assumed to depend on the activity of the phenol hydroxylase, which had been induced during the experiments.

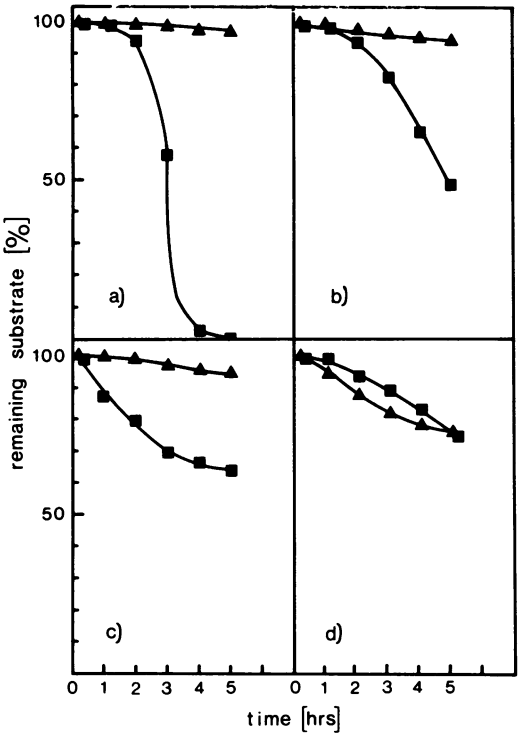

FIG. 2. Turnover of chlorophenols by cells of *Alcaligenes* sp. strain A7-2 and *Pseudomonas* sp. strain B13 growing on 3-chlorobenzoate. Cultures of *Alcaligenes* sp. strain A7-2 (■) and *Pseudomonas* sp. strain B13 (▲) growing exponentially on 3-chlorobenzoate were supplemented with 0.15 mM 4-chlorophenol (a), 0.4 mM 4-chlorophenol (b), 0.15 mM 3-chlorophenol (c), or 0.15 mM 2-chlorophenol (d). The concentrations of the chlorophenols were determined by high-pressure liquid chromatography.

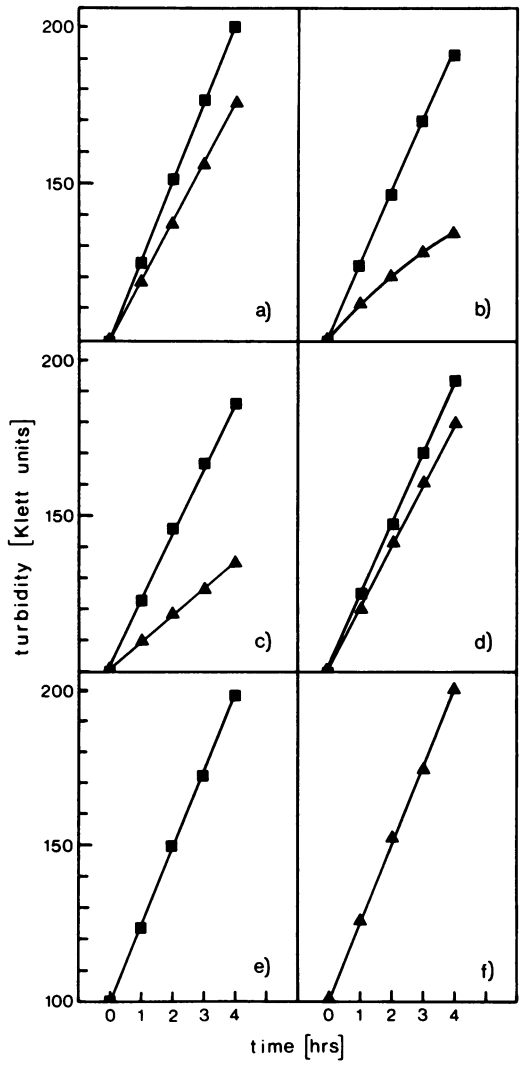

FIG. 3. Growth inhibition by chlorophenols. Cultures of *Alcaligenes* sp. strain A7-2 (■) and *Pseudomonas* sp. strain B13 (▲) growing exponentially on 3-chlorobenzoate were supplemented with 0.15 mM 4-chlorophenol (a), 0.4 mM 4-chlorophenol (b), 0.15 mM 3-chlorophenol (c), or 0.15 mM 2-chlorophenol (d). Control cultures (e) and (f) were run without chlorophenols. Turbidities were measured as Klett units with a Klett-Summerson photoelectric colorimeter.

The increase in turbidity in the flasks supplemented with chlorophenol was compared with that in the control cultures (Fig. 3). 2-Chloro- and 4-chlorophenol considerably inhibited growth of *Pseudomonas* sp. strain B13 when the chlorophenol was present at a concentration of 0.15 mM, whereas 3-chlorophenol nearly totally prevented growth at this concentration. In contrast, the isomeric monochlorophenols did not significantly affect growth of *Alcaligenes* sp. strain A7-2. 4-Chlorophenol was generally tolerated at the high concentration of 0.4 mM in the medium.

Oxidation of phenol and monochlorophenols by whole cells. We investigated whether *Alcaligenes* sp. strain A7-2 is more efficient than *Pseudomonas* sp. strain B13 for the oxidation of chlorophenols. The activities were determined with whole cells by using an oxygen analyzer as described above (Table 2). To compare the activities in *Alcaligenes* sp. strain A7-2 and *Pseudomonas* sp. strain B13, the relative activities are given as percentages of the amount of reaction with phenol. The strains exhibited almost the same relative activities toward chlorinated phenols. However, a comparison of specific activities showed that *Alcaligenes* sp. strain A7-2 induced considerably more of the phenol-oxidizing activity than did *Pseudomonas* sp. strain B13.

TABLE 2. Specific and relative activities of oxidation of phenol and monochlorophenols by whole cells of *Alcaligenes* sp. strain A7-2 and *Pseudomonas* sp. strain B13

Strain	Activity[a] in the following assay substrate:			
	Phenol	4-Chlorophenol	3-Chlorophenol	2-Chlorophenol
Pseudomonas sp. strain B13[b]	37.5 (100)	9.8 (26)	4.0 (11)	4.9 (13)
Alcaligenes sp. strain A7-2[b]	56.9 (100)	18.8 (33)	4.4 (8)	6.6 (12)
Alcaligenes sp. strain A7-2[c]	108 (100)	41.2 (38)	5.0 (5)	17.5 (16)

[a] Specific activities measured by oxygen consumption are expressed as millimoles per liter per gram of protein per minute. Relative activities given within parentheses are percentages of that for phenol.

[b] The cells were grown in continuous culture with 5 mM 4-chlorophenol. The dilution rate was one volume exchange per day.

[c] The cells were grown in continuous culture on a mixture of the three monochlorophenols. The concentration of each monochlorophenol was 2 mM. The dilution rate was one volume exchange per day.

DISCUSSION

Alcaligenes sp. strain A7, which can utilize high concentrations of phenol, acquired halocatechol-degrading capacity from *Pseudomonas* sp. strain B13. However, the transconjugant *Alcaligenes* sp. strain A7-2 appeared only with low frequencies, since phenotypic expression of the utilization of haloaromatics requires not only gene transfer but also a prevention of the *meta* pathway. Ring cleavage of the chlorocatechols by the catechol 2,3-dioxygenase appears to be a dead-end pathway. During oxidation of 4-chlorophenol or 4-chlorocatechol, 5-chloro-2-hydroxymuconic semialdehyde is accumulated (11). 3-Chlorocatechol, the metabolite formed from 2-chlorophenol and mainly from 3-chlorophenol, deactivates the *meta* cleaving activity (8).

The combination of a lower sensitivity to phenols and the halocatechol-degrading capacity resulted in the ability to degrade a mixture of the three isomeric monochlorophenols. In liquid culture *Pseudomonas* sp. strain B13 tolerates only a low concentration of phenol or chlorophenols; therefore, long incubation periods are required before 4-chlorophenol can be utilized in continuous culture (9). This partly explains the inability of the organism to utilize the isomeric chlorophenols. *Pseudomonas* sp. strain B13 is extremely sensitive to chlorophenols, even when the chlorophenol concentration is 0.15 mM. In contrast, growth of *Alcaligenes* sp. strain A7-2 on 3-chlorobenzoate was only slightly inhibited when one of the monochlorophenols was applied at this concentration. Growth of *Pseudomonas* sp. strain B13 was almost completely inhibited by 0.4 mM 4-chlorophenol, whereas 4-chlorophenol at the same concentration was only a very weak inhibitor of cells of *Alcaligenes* sp. strain A7-2.

With the exception of 2-chlorophenol, the chlorophenols induced a phenol hydroxylation activity in *Alcaligenes* sp. strain A7-2 much faster than in *Pseudomonas* sp. strain B13. 4-Chlorophenol is an especially strong inducer for cells of *Alcaligenes* sp. strain A7-2. This activity would lower the concentration of chlorophenols in the cells. Uninduced cells of *Alcaligenes* sp. strain A7-2 also tolerated higher concentrations of chlorophenols than did cells of *Pseudomonas* sp. strain B13, as can be seen from h 1 of the inhibition experiment. In general, *Alcaligenes* sp. strain A7-2 tolerated much higher concentrations of chlorophenols than did *Pseudomonas* sp. strain B13. This partly explains the ability of *Alcaligenes* sp. strain A7-2, in contrast to *Pseudomonas* sp. strain B13, to grow on a mixture of the isomeric chlorophenols. Since the relative oxidation activities toward the chlorophenols were almost the same in both strains, the ability of *Alcaligenes* sp. strain A7-2 to degrade the chlorophenols does not depend on a phenol hydroxylase with higher relative activity for chlorinated phenols. However, the phenol hydroxylase was induced in higher amounts in *Alcaligenes* sp. strain A7-2 than in *Pseudomonas* sp. strain B13.

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