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Aerobic Mineralization of 2,6-Dichlorophenol by *Ralstonia* sp. Strain RK1

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A new aerobic bacterium was isolated from the sediment of a freshwater pond close to a contaminated site at Amponville (France). It was enriched in a fixed-bed reactor fed with 2,6-dichlorophenol (2,6-DCP) as the sole carbon and energy source at pH 7.5 and room temperature. The degradation of 2,6-DCP followed Monod kinetics at low initial concentrations. At concentrations above 300 μM (50 mg · liter^{-1}), 2,6-DCP increasingly inhibited its own degradation. The base sequence of the 16S ribosomal DNA allowed us to assign the bacterium to the genus *Ralstonia* (formerly *Alcaligenes*). The substrate spectrum of the bacterium includes toluene, benzene, chlorobenzene, phenol, and all four *ortho*- and *para*-substituted mono- and dichlorophenol isomers. Substituents other than chlorine prevented degradation. The capacity to degrade 2,6-DCP was examined in two fixed-bed reactors. The microbial population grew on and completely mineralized 2,6-DCP at 2,6-DCP concentrations up to 740 μM in continuous reactor culture supplied with H_2O_2 as an oxygen source. Lack of peroxide completely stopped further degradation of 2,6-DCP. Lowering the acid-neutralizing capacity of the medium to 1/10th the original capacity led to a decrease in the pH of the effluent from 7 to 6 and to a significant reduction in the degradation activity. A second fixed-bed reactor successfully removed low chlorophenol concentrations (20 to 26 μM) with hydraulic residence times of 8 to 30 min.

Chlorinated phenols are widely used by the chemical industry as intermediate products in synthesis and previously were frequently applied as wood preservatives and fungicides (4, 13). Due to their high toxicity, strong odor emission, and persistence in soil these compounds pose serious ecological problems as environmental pollutants (26, 29).

Aerobic degradation of polychlorinated phenols has been studied extensively during the last few years (12). Several strains of bacteria that are able to completely mineralize polychlorinated phenols have been described (5).

Degradation of 2,4-dichlorophenol (2,4-DCP) by pure cultures, especially cultures of *Ralstonia eutropha* (formerly *Alcaligenes eutrophus* [34]) and *Burkholderia cepacia*, has been reported repeatedly (8, 11, 33), as has degradation of 2- and 4-monochlorophenols by *Ralstonia eutropha*, *Pseudomonas putida*, and *Alcaligenes* sp. strain A 7-2 (4, 16, 17). The aerobic 2,4-DCP degradation pathway has been elucidated (6, 9, 25). Degradation of the 2,6 isomer has also been observed in laboratory and field experiments, and transformation of this compound by resting cells of *Mycobacterium chlorophenolicum*, *Azotobacter* sp., and *Pseudomonas cepacia* has been reported (3, 21, 32). Complete degradation of 2,6-DCP in sediment samples has been observed (22). However, no pure culture capable of growing on 2,6-DCP aerobically has been described previously.

In this study we describe the isolation and characterization of a pure culture that grows on 2,6-DCP as a sole source of carbon and energy. The potential of this organism to purify contaminated groundwater or wastewater from soil washing and to remove a series of chlorophenols under nonsterile conditions was studied with fixed-bed reactors. We tested removal rates for short hydraulic residence times (HRTs) for water containing low chlorophenol concentrations, and we also investigated the importance of pH homeostasis and oxygen control for optimal degradation of high concentrations of 2,6-DCP for practical applications.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *Ralstonia* sp. strain RK1 was isolated from a fixed-bed reactor with 2,6-DCP as the sole carbon and energy source. A sample of the reactor effluent was diluted 1:1,000 in a shake flask containing 100 ml of mineral medium supplemented with 120 μM 2,6-DCP. When the 2,6-DCP had been consumed, fresh medium was inoculated with 100 μl of this preculture. This enrichment procedure was repeated four times. A sample from the fifth shake flask was streaked onto nutrient agar plates. Four morphologically distinct types of colonies appeared. One colony of each colony type was picked and streaked onto a separate nutrient agar plate, and a single colony from this plate was removed and incubated in liquid mineral medium supplemented with 2,6-DCP.

(i) Mineral medium. The chloride-free mineral medium used for growth of the pure culture and the experiments with the fixed-bed reactors was designed to correspond to groundwater conditions (28). It consisted of 152 μM $(NH_4)_2HPO_4$, 368 μM KH_2PO_4, 575 μM K_2HPO_4, 0.5 ml of each of the trace element solutions per liter, and 0.5 ml of a vitamin solution per liter. Trace element stock solution 1 contained (per liter) 6.5 mg of $Na_2SeO_3 \cdot 5H_2O$ and 12.1 mg of $Na_2WO_4 \cdot 2H_2O$ in 0.1 N NaOH, and trace element stock solution 2 contained (per liter) 2,250 mg of $FeSO_4 \cdot 7H_2O$, 90 mg of H_3BO_3, 150 mg of $MnSO_4 \cdot H_2O$, 210 mg of $CoSO_4 \cdot 7H_2O$, 55 mg of $NiSO_4 \cdot 6H_2O$, 150 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of $CuSO_4 \cdot 5\ H_2O$, and 80 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 0.001 N H_2SO_4. The vitamin solution contained (per liter) 2 mg of biotin, 20 mg of nicotinic acid, 10 mg of thiamine, 10 mg of 4-aminobenzoate, 5 mg of pantothenate, 20 mg of cyanocobalamin, and 50 mg of pyridoxamine; it was filter sterilized and added after autoclaving.

The mineral medium had a total salt concentration of 1.1 mM, the electric conductivity was 220 μS · cm^{-1}, and the acid-neutralizing capacity was 182 mM equivalents of H^+ at pH 7.5. This medium corresponded well to the groundwater conditions at the Amponville site (pH 7.6; conductivity, 320 μS · cm^{-1}).

(ii) Complex medium. Complex medium contained 10% nutrient broth and was prepared by using the specifications of the supplier (Difco).

Microscopy. Morphological examinations were carried out by phase-contrast microscopy (model M-20 microscope; Wild, Heerbrugg, Switzerland) and scan-

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TABLE 1. Taxonomic features of genera and species related to *Ralstonia* sp. strain RK1

Taxon	Gelatin lysed	Nitrate reductase	Acid formed from glucose	Growth on D-glucose	Growth on gluconate	Growth on adipate	Pigment excretion	Flagellum(a)
Ralstonia sp. strain RK1	−[b]	−	−	−	+	+	−	−
Ralstonia eutropha[a]	−	+	−	−	+	+	−	+
Alcaligenes faecalis[a]	−	−	−	−	−	−	−	+
Burkholderia[a]	d	+	−	+	+	+	+	+
Pseudomonas pickettii[a]	−	+	+	+	+	+	−	+

[a] Data from reference 18.
[b] −, less than 10% of all strains tested are positive; +, more than 90% of all strains tested are positive; d, between 10 and 90% of all strains tested are positive.

ning electron microscopy (model S-4000 microscope; Hitachi, Tokyo, Japan). For the latter, cells were harvested by centrifugation at 3,000 × *g*, washed twice with mineral medium, and fixed with 3% glutaraldehyde. Then the medium was gradually changed to 100% acetone, cells were transferred to a polycarbonate filter, and critical point drying was carried out with CO_2 and sputtering with argon.

Taxonomic determination. Physiological and biochemical tests were carried out with an API 20NE kit (BioMerieux, Marcy l'Etoile, France). Cells grown on complex medium were used to sequence the 16S ribosomal DNA. The total DNA was obtained by phenolic extraction (27). The RNA was removed by incubating an aqueous solution for 15 min at 37°C with 5 U of DNase-free RNase. A PCR with the extracted DNA was performed with two eubacterial primers, S-D-Bact-0008-a-S-20 (15) and S-D-Bact-1492-a-A-19 (20). The designations of the primers were standardized as described by Alm et al. (1). Amplification was carried out with a thermocycler (Techne, Duxford, United Kingdom) by using a 25-μl sample. Each reaction tube contained *Pfu* reaction buffer, 1.5 mM $MgCl_2$, 100 μM deoxynucleotide triphosphate, 1 U of *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), 5 pmol of each primer, and 100 ng of template DNA. The PCR steps included initial denaturation at 95°C for 3 min, 25 cycles consisting of 95°C for 1 min, 50°C for 1 min, and 72°C for 2 min, and a final extension step consisting of 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel. The bands expected for the 16S ribosomal DNA were cut out, and the DNA was purified by phenolic gel extraction as described previously (27). Sequencing of the 16S rRNA gene was carried out with a model ABI 310 Prism Collector (Perkin-Elmer, Foster City, Calif.) according to the supplier's instructions. The following primers were used for the partially double-stranded sequencing analysis: S-D-Bact-0008-a-S-20 (15), S-D-Bact-0338-a-A-18 (2), *-Univ-0519-a-S-18, *-Univ-0519-a-A-18, *-Univ-1392-a-A-15 (24), and S-D-Bact-1492-a-A-19 (20).

A computer analysis was carried out with the University of Wisconsin Genetics Computer Group software packages, version 8. The sequence of the 16S rRNA of the 2,6-DCP-degrading bacteria was compared with other available 16S rRNA sequences by using the FASTA search option of the EMBL database to determine the closest phylogenetic neighbors.

Growth experiments. Growth experiments were performed in duplicate in 500-ml shake flasks containing 100 ml of mineral medium and, unless stated otherwise, 20 mg of the corresponding test substance per liter. The precultures used for inoculation were grown for 3 days on 120 μM 2,6-DCP (20 mg of 2,6-DCP per liter). The concentration of the remaining 2,6-DCP was below the quantification limit, 1.8 μM. A 0.1-ml aliquot (1‰) of the preculture was transferred into new medium, and growth was allowed to proceed at 20°C on a rotary shaker at 144 rpm. Both substrate disappearance and cell growth to at least 10 times the inoculum concentration were used as criteria for growth.

Reactor setup. The fixed-bed reactors consisted of glass columns with an inner diameter of 3 cm and a total volume of 210 ml that were filled with 120 ml of sintered glass beads (diameter, 2 to 4 mm; Siran; Schott, Mainz, Germany). The reactors were run with mineral medium (pH 7.5) in upstream mode.

The flow rate of reactor 1 was 750 ml · day^{-1}, which yielded an HRT of 3.84 h as related to the interstitial volume of the fixed bed. The 2,6-DCP feed concentration was gradually increased from 250 to 740 μM. Simultaneously, the acid neutralization capacity and the trace element concentration of the mineral medium were increased up to threefold. Hydrogen peroxide (30%, vol/vol) was diluted to a molar ratio of about 12:1 in relation to the actual 2,6-DCP concentration to provide enough oxygen for complete mineralization of the substrate. Sufficient oxygen was indicated by oxygen effluent concentrations of 1 to 8 mg · $liter^{-1}$.

Reactor 2 was run with constant feed concentrations of 2,4-DCP and 2,6-DCP (9 μM each) and 2,4,6-trichlorophenol (2,4,6-TCP) (7.6 μM). The flow rate was gradually increased from 6 to 78 liters · day^{-1}, which yielded HRTs between 0.48 and 0.04 h.

Analytical methods. Aromatic compounds were analyzed by reversed-phase high-performance liquid chromatography performed with a model 300 system (Kontron Instruments, Everett, Mass.) equipped with a Nucleosil C_{18} column (Bischoff, Leonenberg, Germany). Chlorophenols were separated by using a 30 to 90% acetonitrile gradient (pH 2 adjusted with H_3PO_4). The flow rate was 1 ml · min^{-1}, and data were quantified by absorbance at 290 nm. The typical retention time for 2,6-DCP was 8.7 min. The quantification limits were 1.8 μM for 2,6-DCP and 0.6 μM for 2,4-DCP and 2,4,6-TCP. The chloride ion concentration was determined with the Spectroquant system (catalog no. 1.14755; Merck, Darmstadt, Germany), and hydrogen peroxide was measured with the Reflectoquant peroxide test system (catalog no. 16974; Merck). The glucose concentration was determined with Trinder reagent (Sigma Chemical Co., St. Louis, Mo.) by using the supplier's instructions. Proton activity was measured with a portable pH meter (Portamess 751; Knick, Darmstadt, Germany). CFU were determined by direct plating following 3 days of incubation on 1.5% agar (Bacto Agar and nutrient agar; Difco Laboratories, Detroit, Mich.) amended with 0.3‰ beef extract and 0.5‰ peptone. The optical densities of liquid cultures were measured at 546 nm.

Chemicals. The chemicals used in this study were analytical grade. 3,5-DCP and 2,3,5,6-tetrachlorophenol were purchased from Aldrich-Europe, Beerse, Belgium; acetonitrile, *ortho*-phosphoric acid, 2,4,6-TCP, 3,4-DCP, 2,6-DCP, toluene, and tetrachloroethylene were obtained from Merck. All other chemicals were purchased from Fluka, Buchs, Switzerland.

Nucleotide sequence accession number. The 16S rRNA sequence of strain RK1 has been deposited in the EMBL nucleotide sequence database under accession no. AJ002302.

RESULTS

Taxonomic characteristics. Strain RK1 was the only isolate from fixed-bed reactor 1 that was able to grow in pure culture on 2,6-DCP as the sole carbon and energy source. This organism is a white-pigmented, gram-negative, oxidase- and catalase-positive rod that is 1.5 to 4 μm long and 0.6 to 0.8 μm wide. No extracellular structures and no spores are visible when preparations are examined by light microscopy or scanning electron microscopy. Small, white, transparent, circular colonies with smooth surfaces can be observed after incubation for 2 days at room temperature on nutrient agar plates. The colonies change to a flowerlike shape when plates are incubated for longer periods of time (up to 5 days). The bacterium is urease positive and arginine dihydrolase and β-galactosidase negative; it lyses neither esculin nor gelatin, cannot reduce nitrate, is not able to grow anaerobically, and does not form acids from sugars. It is able to grow on the following substrates: gluconate, caprate, adipate, malate, citrate, succinate, pyrocatechol, benzene, toluene, chlorobenzene, phenol, 2-chlorophenol (2-CP), 4-CP, 2,4-DCP, and 2,6-DCP. It does not grow on glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, 3-CP, 2,3-DCP, 3,4-DCP, 3,5-DCP, any isomer of trichlorophenol, 2,3,5,6-tetrachlorophenol, pentachlorophenol, 2,4-dichlorophenoxyacetic acid, 1,3-dichlorobenzene, 1,2,3-trichlorobenzene, 2,6-dichlorotoluene, 2,6-dinitrophenol, 2,6-dimethylphenol, or 1,2,3-trichloropropane. Important taxonomic features of strain RK1 and its closest phylogenetic neighbors are listed in Table 1.

Sequencing of the 16S rRNA gene and comparison with previously published 16S rRNA gene sequences resulted in classification of strain RK1 as a member of the genus *Ralstonia*. The highest degree of similarity found was 97.3%, which was the value obtained with the 16S rRNA gene of an *Alcaligenes* sp. (Table 2). *Ralstonia* sp. strain RK1 has been deposited in the Deutsche Sammlung von Mikroorganismen und

TABLE 2. Species related to *Ralstonia* sp. strain RK1

Taxon	EMBL accession no.	% 16S rRNA homology with *Ralstonia* sp. strain RK1
Ralstonia sp. strain RK1	AJ002302	100
Alcaligenes sp.	L31650	97.3
Ralstonia eutropha	M32021	95.7
Pseudomonas solanacearum	X67035	93.3
Pseudomonas pickettii	L37367	92.7
Burkholderia sp.	X92188	90.7
Alcaligenes faecalis	D88008	86.8

Zellkulturen GmbH, Braunschweig, Germany, as strain DSM 11853.

Growth characteristics. *Ralstonia* sp. strain RK1 completely degrades 2,6-DCP under oxic conditions, and chloride ions are produced stoichiometrically (Fig. 1A). No UV-detectable intermediate products were detected in the fixed-bed reactor experiments and in batch assays with substrate concentrations below 1,200 μM. The bacterial cell yield was 119 ± 34 CFU · pmol of 2,6-DCP^{-1} and was constant for initial 2,6-DCP concentrations greater than 120 μM; the bacterial cell yield was slightly higher for lower concentrations (data not shown). The yield obtained corresponds to about 7.5 g of protein · mol of C^{-1}, assuming a weight of 1 μg/microorganism and a protein content of 50% (wt/wt) (31). The high protein yield, the consumption of approximately 12 mol of hydrogen peroxide (yielding 6 mol of O_2) per mol of 2,6-DCP in fixed-bed reactor 1, and the fact that no aromatic or other conjugated intermediates were detected justify the assumption that 2,6-DCP was completely mineralized.

At concentrations greater than 1,200 μM, degradation of 2,6-DCP was not complete, and the culture fluid turned purple. High-performance liquid chromatography analysis revealed a series of unidentified aromatic substances, while the UV spectrum of the liquid culture had a peak at 290 nm and a second, large, flat peak at 530 nm.

The lag phase increased exponentially with increasing initial substrate concentration (Fig. 1B). The maximal growth rate (0.076 h^{-1}) was reached when the 2,6-DCP concentration was approximately 300 μM, and the growth rate decreased rapidly with higher substrate concentrations (Fig. 1C). The maximal growth rate, K_S, and K_i were determined by Lineweaver-Burk linearization (23) of the data shown in Fig. 1C. At initial 2,6-DCP concentrations less than 300 μM, the growth rate followed Monod kinetics, with a maximal growth rate of 0.082 h^{-1} and a K_S of 24.1 μM. The decrease in the growth rate at higher initial concentrations was attributed to inhibitory effects of 2,6-DCP. The decreasing part of the curve could be described by the Haldane equation (14). Neither the Monod model nor the Haldane model could be used to describe the growth rate over the whole concentration range, however.

Since nonsterile conditions and mixed bacterial cultures are prevalent in field applications, we compared the performance of the pure culture of strain RK1 with the performance of a mixed culture obtained from an aliquot from a fixed-bed reactor (reactor 1) (see below). The mixed culture was transferred five times in fresh mineral medium containing 120 μM 2,6-DCP as described above for the isolation of *Ralstonia* sp. strain RK1. The resulting mixed culture had a bacterial cell yield of 172 ± 7 CFU · pmol of 2,6-DCP^{-1} and a growth rate of 0.15 h^{-1} at an initial 2,6-DCP concentration of 120 μM (data not shown). *Ralstonia* sp. strain RK1 was the only isolate obtained from reactor 1 that grew on 2,6-DCP as a sole carbon and energy source. More than 90% of all colonies from reactor 1 that grew on complex medium were morphologically similar to *Ralstonia* sp. strain RK1 colonies. We assumed, therefore, that *Ralstonia* sp. strain RK1 accounted for a major portion of the reactor biomass and that it grows well and maintains a metabolically active population under nonaseptic conditions.

Reactor experiments. Reactor experiments with two different reactors were conducted to determine whether mixed cultures could be used to remove chlorophenols from water and soil. Both reactors were inoculated with sediment samples from a freshwater pond at the Amponville site, which is contaminated with 2,4-DCP, 2,6-DCP, and 2,4,6-TCP.

Fixed-bed reactor 1 was run with high concentrations of 2,6-DCP (220 to 740 μM) to investigate the feasibility of treating wastewater from soil washing. The reactor was allowed to adapt for 55 days to 220 μM 2,6-DCP before the feed concentration was increased stepwise to 740 μM over a period of 22 days (Fig. 2, arrow 1). This led to an almost stoichiometric increase in chloride ions in the outlet. At day 92 (arrow 2), the addition of hydrogen peroxide was interrupted, and mineralization ceased almost instantaneously. Then 11 days later, hydrogen peroxide was added again, and simultaneously the concentration of 2,6-DCP was lowered to 400 μM (arrow 3). 2,6-DCP degradation and chloride release resumed quickly, and the feed concentration was readjusted to its former value. Lowering the acid-neutralizing capacity of the medium to 1/10th the initial capacity (arrow 4) led to a decrease in the pH

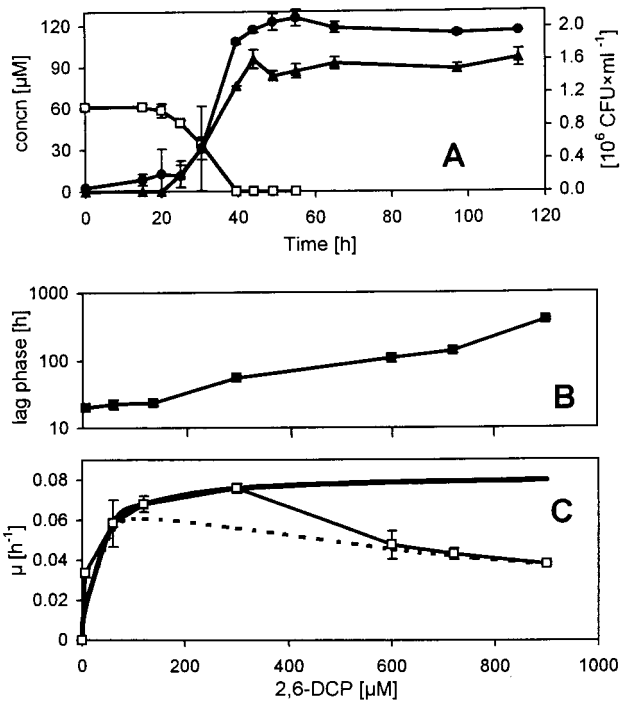

FIG. 1. Growth characteristics of *Ralstonia* sp. strain RK1 on 2,6-DCP as determined with batch cultures. (A) Mineralization of 60 μM 2,6-DCP by *Ralstonia* sp. strain RK1 in a batch culture. Symbols: □, 2,6-DCP concentration; ●, chloride ion concentration; ▲, CFU per milliliter. (B) Lag phase of *Ralstonia* sp. strain RK1 with increasing initial concentrations of 2,6-DCP. (C) Growth rate of *Ralstonia* sp. strain RK1 at different initial concentrations of 2,6-DCP. Solid line, curve obtained with the Monod equation (23) with a maximal growth rate of 0.082 h^{-1} and a K_s of 24.1 μM obtained by Lineweaver-Burk linearization of the first five data points; dashed line, prediction from Haldane equation (Monod with inhibition; K_i = 787 μM, as determined by linear regression of the last four data points). μ, growth rate. Error bars represent standard deviations from two cultures. The same *x* axis applies to panels B and C.

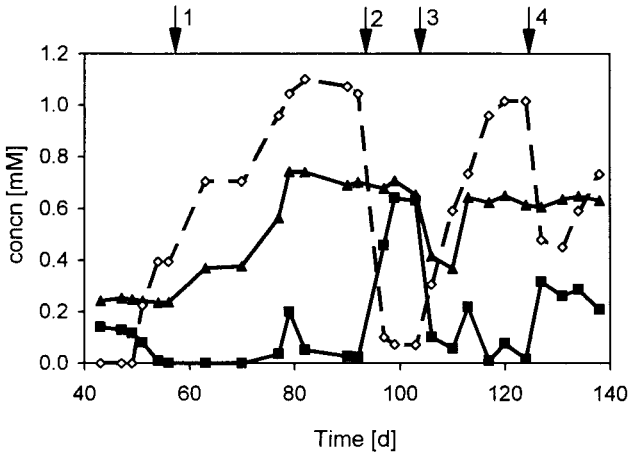

FIG. 2. Mineralization of high concentrations of 2,6-DCP in fixed-bed reactor 1. Symbols: ▲, 2,6-DCP feed concentration; ■, 2,6-DCP effluent concentration; ◇, chloride ion effluent concentration. The arrows indicate experimental changes described in the text. d, days.

from 7 to 6 in the effluent (data not shown). Concomitantly, the performance of the reactor decreased significantly. It took 10 days before the system began to adapt to the lower pH and reactor performance increased again.

Fixed-bed reactor 2 was run with a mixture of 2,4-DCP, 2,6-DCP, and 2,4,6-TCP at low concentrations (9.2, 9.2, and 7.5 μM, respectively) in order to simulate conditions in contaminated groundwater. As *Ralstonia* sp. strain RK1 does not degrade 2,4,6-TCP, complete removal of this compound at a low HRT was performed by other members of the mixed bacterial population of the reactor. Decreasing the HRT stepwise from 28.8 to 2.4 min led to a transient breakthrough of 2,4,6-TCP at an HRT of 18 min and transient breakthroughs of 2,4-DCP and 2,6-DCP at an HRT of 6 min (Fig. 3A and B). Despite the breakthroughs, only the initial chlorophenols were detected in the effluent; no transformation products were detected. Keeping the HRT constant for several days resulted in a decrease in the chlorophenol concentration in the effluent even at very high flow rates. At HRTs of less than 3.6 min, the conversion coefficient dropped below 0.7 and remained below this value for the remainder of the experiment (Fig. 3C). With reactor 2, the volumetric degradation yield reached 1.2 g · liter^{-1} · day^{-1} with a conversion coefficient as high as 0.85. The maximal volumetric degradation yield achieved in this experiment was as high as 1.6 g · liter$_R^{-1}$ · day^{-1} (Fig. 3C).

DISCUSSION

Degradation of chlorophenols has been reported very often. *Ralstonia* sp. strain RK1 is the first pure culture capable of growing on and mineralizing 2,6-DCP.

The 16S rRNA gene of the newly isolated bacterium described in this paper exhibits the highest levels of homology with the 16S rRNA genes of members of the genera *Alcaligenes* and *Burkholderia* which have been transferred to the genus *Ralstonia* (34). According to Stackebrandt and Goebel (30), the generally accepted limit for a relationship at the species level is 70% DNA similarity, and DNA similarities of more than 60% are very unlikely to occur with 16S rRNA homologies of 97% or less. Since (i) the most closely related *Alcaligenes* sp. has not been identified to the species level (Table 2), (ii) the levels of 16S rRNA homology between strain RK1 and the three previously described *Ralstonia* species (*Ralstonia eutropha*, *Ralstonia solanacearum*, and *Ralstonia pickettii*) are less than 96% (Table 2), and (iii) the new *Ralstonia* sp. strain has a different phenotype than the previously described species of the genus *Ralstonia* (Table 1), we suggest that the new strain should be placed in a new species in the genus *Ralstonia* and that strain RK1 should be regarded as the type strain. We propose the name *Ralstonia basilensis* (from *basilea*, Latin for Basel, Switzerland, where the strain was isolated) for this organism.

The substrate spectrum of strain RK1 includes nonhalogenated aromatic compounds, like benzene, toluene, and phenol, as well as chlorobenzene and all mono- and dichlorophenols with a chloride substituent(s) in the *ortho* and/or *para* position.

Hydroxylation in the *ortho* position, which produces chlorocatechol, has been reported to be the first step in the aerobic degradation of mono- and dichlorophenols (9, 12). The initial steps used by *R. basilensis* RK1 for ring cleavage must be different from the pathway described by Don et al. (9). 2,4-DCP hydroxylase cannot act on the *ortho* positions of 2,6-DCP, because both of these positions are occupied by chlorine atoms. In addition, *R. basilensis* RK1 also lacks the ability to degrade

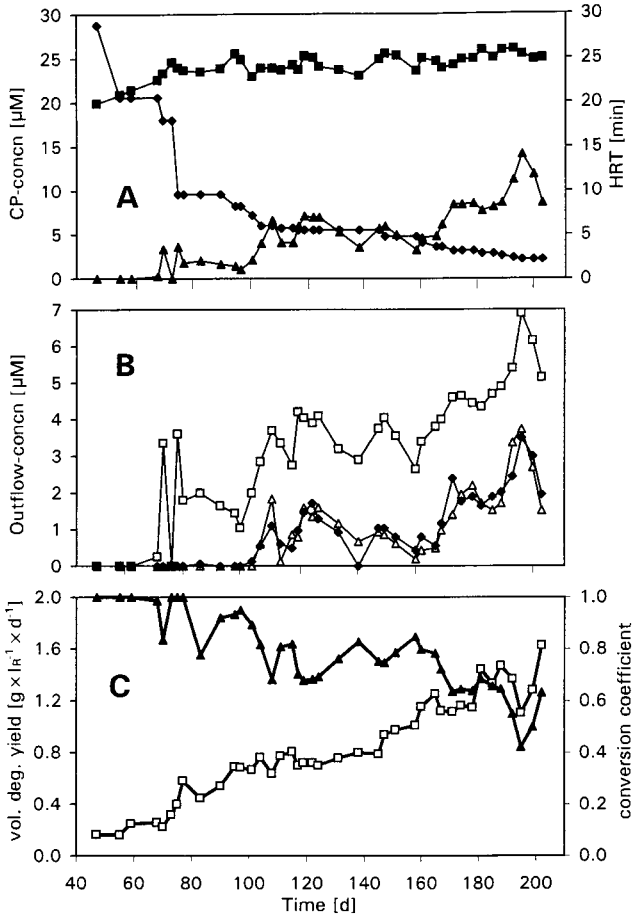

FIG. 3. Mineralization of low concentrations of 2,4-DCP, 2,6-DCP, and 2,4,6-TCP with short HRTs in fixed-bed reactor 2. (A) Symbols: ■, feed concentration of chlorophenols (CP); ▲, effluent concentration of chlorophenols; ◆, HRT. (B) Effluent concentrations of the three chlorophenol congeners. Symbols: □, 2,4,6-TCP; △, 2,6-DCP; ◆, 2,4-DCP. (C) Reactor performance. Symbols: □, volumetric degradation (vol. deg.) yield; ▲, conversion coefficient [(feed concentration − outflow concentration)/feed concentration]. The same *x* axis applies to panels A through C. d, days; lR, liter reactor volume.

2,4-dichlorophenoxyacetic acid. Further investigations are in process to evaluate the 2,6-DCP degradation pathway.

Lengthening of lag phases with increasing initial substrate concentrations may be interpreted as a consequence of metabolic inhibition by 2,6-DCP at concentrations above 300 μM, since 2,6-DCP and other chlorophenols can act as uncoupling agents in biological membranes (7, 21, 29). Escher et al. (10) reported that the concentration required to inhibit the NAD^+ reduction rate by 50% was 8.9 mM for 2,6-DCP. In our experiments, which were performed with concentrations that were 10 times lower, the bacterial cell yield remained unchanged, while the lag phase increased from up to 3 days at 2,6-DCP concentrations below 300 μM to 16 days at 1,200 μM 2,6-DCP. The prolonged lag phase at high initial 2,6-DCP concentrations may reflect slow growth of the bacteria due to large energy dissipation from simultaneous chlorophenol degradation and uncoupling. The lower growth rates at high initial 2,6-DCP concentrations also reflect substrate inhibition.

Under comparable conditions, a mixed culture from reactor 1 degraded 2,6-DCP faster and with a higher cell yield than a pure culture of *R. basilensis* RK1. Our observation that the maximum growth rate of an enriched, specialized pure culture is only one-half the maximum growth rate of a mixed culture (0.076 versus 0.15 h^{-1}) is in contrast to observations made with phenol-degrading strains of *Acinetobacter calcoaceticus* and *Pseudomonas fluorescens* (19). There are several possibilities which might account for our experience. Community synergism based on 2,6-DCP products might prevent the buildup of metabolic intermediates and thus minimize feedback regulation of the consumption of 2,6-DCP. Alternatively, the community might contain other bacterial populations that are able to degrade 2,6-DCP, which we were not able to isolate on nutrient agar plates.

With the fixed-bed reactors used in this study we were able to demonstrate the practicability of eliminating chlorophenols in water through biological degradation. The ability to degrade high concentrations of 2,6-DCP demonstrated with reactor 1 opens up new possibilities for treating wastewater from chemical soil washing without further dilution. Elimination of 46 mmol of 2,6-DCP per liter of reactor volume per day (at a 2,6-DCP concentration of 720 μM) consumed 750 ml of mineral medium, 62 ml of hydrogen peroxide (30%), and very little energy for pumping. With fixed-bed reactor 2, we treated a simulated groundwater containing low concentrations of 2,4-DCP, 2,6-DCP, and 2,4,6-TCP. The concentrations of all three isomers were reduced to below the detection limits with an HRT of 20 min and were reduced 95% with an HRT of 8.2 min. Longer adaptation times might improve reactor performance.

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