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Degradation of polychlorinated phenols by *Streptomyces rochei* 303

Ludmila A. Golovleva, Olga Zaborina, Raisa Pertsova, Boris Baskunov, Yuri Schurukhin & Sergei Kuzmin
Institute of Biochemistry and Physiology of Microorganisms, Academy of Sciences, Pushchino, Moscow Region, 142292, Russia

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Abstract

The strain *Streptomyces rochei* 303 (VKM Ac-1284D) is capable of utilizing 2-chloro-, 2,4-, 2,6-dichloro- and 2,4,6-trichlorophenols as the sole source of carbon. Its resting cells completely dechlorinated and degraded 2-, 3-chloro-; 2,4-, 2,6-, 2,3-, 2,5-, 3,4-, 3,5-dichloro-; 2,4-, 2,6-dibromo-; 2,4,6-, 2,4,5-, 2,3,4-, 2,3,5-, 2,3,6-trichlorophenols; 2,3,5,6-tetrachloro- and pentachlorophenol. During chlorophenol degradation, a stoichiometric amount of chloride ions was released and chlorohydroquinols were formed as intermediates. In cell-free extracts of *S. rochei,* the activity of hydroxyquinol 1,2-dioxygenase was found. The enzyme was induced with chlorophenols. Of all so far described strains degrading polychlorophenols, *S. rochei* 303 utilized a wider range of chlorinated phenols as the sole sourse of carbon and energy.

Abbreviations: CP – chlorophenol; DCP – dichlorophenol; TCP – trichlorophenol; TeCP – tetrachlorophenol; PCP – pentachlorophenol; DBrP – dibromophenol; CHQ – chlorohydroquinol; DCHQ – dichlorohydroquinol; HHQ – hydroxyhydroquinol; CHHQ – chlorohydroxyhydroquinol; CC – chlorocatechol; TLC – thin layer chromatography; GC/MC – chromato-mass-spectrometry; HPLC – high-performance liquid chromatography

Introduction

Nowadays, polychlorophenols are among the most persistent pollutants of the environment. Considerable progress has been made in the isolation and investigation of bacterial strains which degrade chlorophenols (Chu & Kirch 1973; Saber & Crawford 1985; Apajalachti & Salkinoja-Salonen 1986). The pathways of their degradation are closely investigated. Two routes of chlorophenol degradation are known: via formation of chlorocatechols and via production of hydroxyhydroquinol. In the first case, there occurs the dechlorination of the aliphatic intermediates after the ring cleavage; in the second, the initial substrate and its metabolites are dechlorinated (before the ring cleavage). The former pathway is known mainly for mono- and dichlorophenols. The key enzyme of chlorocatechol ring cleavage is pyrocatechase II (Knackmuss & Hellwig 1978; Spain & Gibson 1988; Gorlatov et al. 1989). The other route was shown in the degradation of tri-, tetra- and pentachlorophenols (Apajalachti & Salkinoja-Salonen 1986, 1987a, b; Häggblom et al. 1988; Chu & Kirsch 1973; Saber & Crawford 1985). The intermediates of this route of chlorophenol decomposition are chlorohydroquinols and hydroxyhydroquinol. The key enzymes for degradation of the latter compound were not investigated in chlorophenol utilization works; however, earlier it has been shown (Gall & Neu-

jahr 1979; Chapman & Ribbons 1976; Sze & Dagley 1984) that 1,2,4-trihydroxybenzene can be cleaved by both pyrocatechase (Gaal & Neujahr 1979) and hydroxyhydroquinol 1,2-dioxygenase (Chapman & Ribbons 1976; Sze & Dagley 1984).

We isolated a bacterial culture which degraded a broad range of chlorophenols – from mono- to pentachlorophenol.

The purpose of this work was to study the pathways of decomposition of chlorophenols by the culture and to assay the activities of the key enzymes of their degradation.

Materials and methods

Microorganism

Polychlorophenol-degrading strain 303 was isolated from soil by the enrichment culture technique with 2,4-dichlorophenol as the sole source of carbon.

The strain grown on most media formed the well-developed sporulating aerial mycelium of grey colour. The spore chains are spiral; the spore surface is smooth.

The cell wall contains diaminopimelic acid. The study of the physiologo-biochemical characteristics showed that strain 303 can utilize cellobiose, fructose, inositol, sucrose, galactose, lactose, maltose, mannitol, *L*-rhamnose, xylose as the sole source of carbon; does not use raffinose, adonitol, erythritol, *D*-arabinose; uses *L*-histidine, hydroxyproline and (poorly) α-amino-butyric acid as the nitrogen source; hydrolyzes pectin, lecithin, arbutin; forms H_2S; possesses a nitroreductase activity; grows on media with 7% NaCl, 0.01% NaN_3, 0.1% phenol; grows at 45° C, optimal temperature 29–30° C. Resistant to neomycin at a concentration of 50 μg/ml, sensitive to rifampicin at 50 μg/ml.

The strain possesses no antibiotic activity with respect to *Aspergillus niger, Bacillus subtilis, Streptomyces murinus*. By these characteristics, it is similar to the predominant majority of the cluster of the species *Streptomyces rochei* (Williams et al. 1983).

Culture conditions

The mineral medium employed for cultivation did not contain chloride ions and was composed of (g/l): Na_2SO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.8; KH_2PO_4, 0.7; $(NH_4)_2HPO_4$, 1.5. To obtain the agarized medium, 1.5% agar (Difco) was added. The medium was sterilized at 120° C for 30 min and then supplemented with 2-CP; 2,4-, 2,6-DCP; 2,4,6-TCP as the sodium salt to a final concentration of 10 to 400 ppm. The solutions of chlorophenols were subjected to sterilization through a 0.22 μM membrane filter (Millipore). The culture for inoculum was grown for 7 days on the agarized medium with the respective chlorophenol (50 ppm). Cultivation was carried out in Ehrlenmeyer flasks containing 100 ml medium each at 29° C on a rotary shaker (180 rpm).

In induction experiments, the mineral medium with succinate (1 g/l) was used. At 6 h and 23 h after starting the incubation the respective chlorophenol was added to a final concentration of 20 ppm. The biomass was harvested after 30 h of growth.

Incubation with resting cells

The cells grown in the medium with succinate and 2,4,6-TCP were centrifuged, washed twice with 50 mM sodium phosphate buffer (pH 7.0) and suspended in the same buffer. The suspension (50 ml) contained 2,0 g (wet weight) of cells and 0.5, 1.0, 2.5, 5.0 mg of one of the above substrates: 2-, 3-, 4-CP; 2,4-, 2,3-, 2,5-, 2,6-, 3,4-, 3,5-DCP; 2,4,6-, 2,3,4-, 2,4,5-, 2,3,5-, 2,3,6-TCP; 2,3,5,6-TeCP, PCP; 2, 4-, 2,6-DBrP. The concentrations of chloride ions and chlorophenols were determined in the samples taken after 24 h incubation.

Assays with labelled water were carried out similarly, using phosphate buffer (pH 7.0) prepared on $H_2{}^{18}O$ (content of $H_2{}^{18}O$, 81.05%). 2,4-DCP was used as substrate. Aliquots were taken after 3, 6, 12 and 24 h of incubation.

The reaction mixture without cells served as control in all assays; in labelled water experiments, an additional control with CHQ was done.

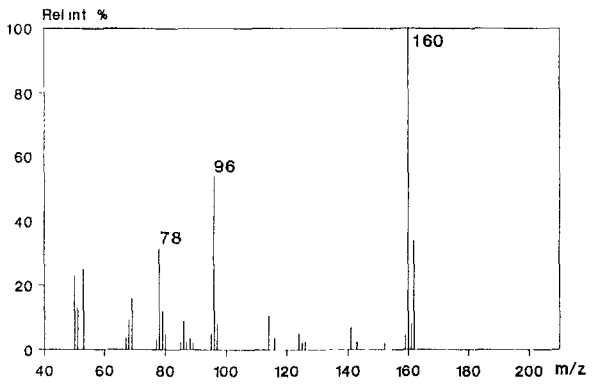

Fig. 1. Mass spectrum of synthesized 6-chloro-2-hydroxyhydroquinol.

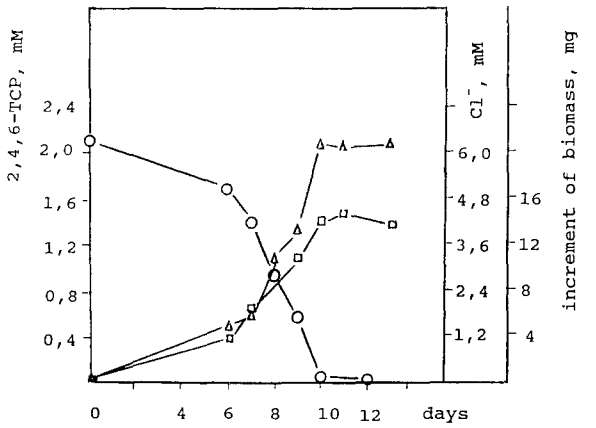

Fig 2. Utilization of 2,4,6-TCP by the culture *S. rochei* 303. ○ 2,4,6-TCP. △ release of chloride. □ increment of biomass.

Preparation of cell-free extract

The required aliquot of cells was suspended in 2–3 volumes of 50 mM Na-phosphate buffer, containing 5 mM dithiothreitol, and supplemented with 25 ml of 0.1 M solution of $MgSO_4$ containing 5 μg/ml of DNAse in 1 ml of cell suspension. Cells were disrupted by extrusion from the solid state at an IBFM press (working pressure in the disintegration chamber, 3500 kg/cm^2; temperature, $-40°$ C; extrusion slot, 350 μm). The nondisrupted cells were removed by centrifugation at 7000 *g* for 30 min. The supernatant was used in further work.

Analytical methods

The concentration of chloride was determined by a chloride-selective electrode on an EA940 Ion Analyzer (Orion Research Inc., Cambridge MA, USA) and by the mercury (II) thiocyanate method (Florence et al. 1971).

Isolation of metabolites

The culture liquid supernatant was extracted three times with an equal volume of diethyl ether with acidification with HCl to pH 2.0. The extracts were dried by dehydrated Na_2SO_4. The solvent was evaporated in a rotor evaporator. TLC was done on Silufol UV-254 plates (Kavalier, Czechoslovakia) in a system consisting of benzene: dioxane:acetic acid at a ratio of 90:10:2 (v/v).

Chlorinated compounds were revealed using silver nitrate (Szokolay & Madaric 1969); phenolic substances were detected by diazobenzidine (Kirchner, 1978).

GC/MS was carried out at a 2091 instrument (LKB Instruments Inc., Bromma, Sweden) at an accelerating voltage of 3.5 kV. The chromatographic conditions were as follows: a glass column, 180 × 0.2 cm filled with Chromosorb W/HP sorbent with 2% phase OV-101. The initial temperature of the column, 100° C; the rate of programmable heating, 10° C/min. The compounds were identified by comparing their retention times and mass spectra with the standards. To analyze the polar products, the culture liquid extracts were treated with diazomethane (Schlenk & Hellerman 1960). HPLC (a system consisting of a 2150 LKB pump, a 7125 injector, a 2151 wavelength monitor, a 2221 integrator; LKB Instruments Inc., Bromma, Sweden) was carried out on a reversed-phase column (4.0 × 250 mm, Spherisorb ODS-2, 5 μm, 2134 LKB) at 280 nm. The metabolites were eluted with a system of solvents 5 mM KH_2PO_4 (adjusted to pH 2.0 with analytical grade concentrated H_2SO_4): methanol at the following ratios: I, 30:70; II, 50:50; III, 20:80 (v/v).

The solvent flow rate was 0.7 ml·min^{-1}. Quanti-

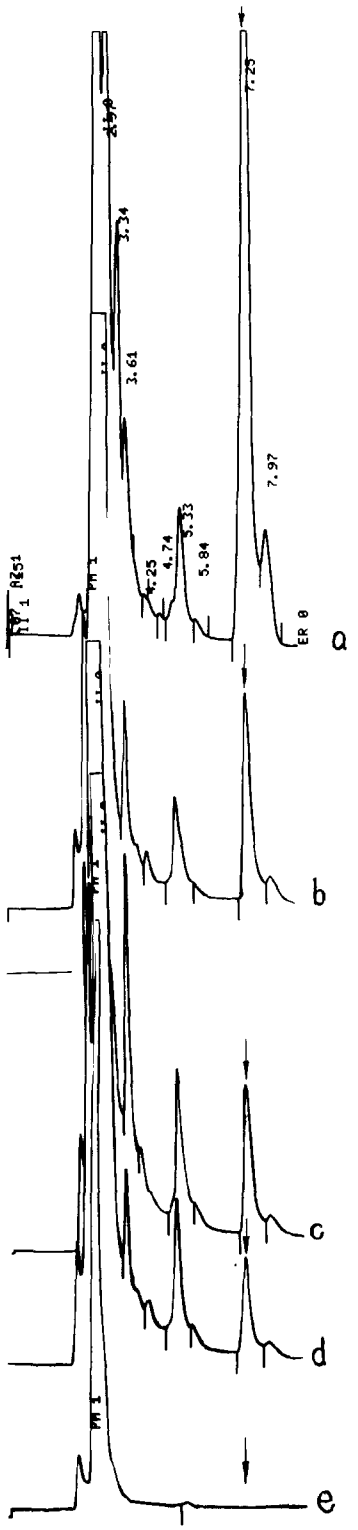

Fig. 3. Degradation of PCP by *S. rochei* 303 resting cells (↓ PCP; a, initial amount; b, in 1.5 h; c, in 2.5 h; d, in 6 h; e, in 17 h).

tative determination of chlorophenols was performed in system III using the method of external standards, and also by UV absorbance determinations by Specord M40 (Karl Zeiss, Jena) using a standard curve.

The amount of biomass was determined by dry weight.

The dehalogenase activity was established by disappearance of the substrate and release of chloride (Fetzner er al. 1989). The reaction mixture contained (in the final volume of 3.5 ml) 50 mM sodium-phosphate buffer pH 7.0, 1.8 mM NADH, 1 ml cell-free extract (containing 4–6 mg of protein) and 0.15 mM of respective chlorophenol. Aliquots of 0.6 ml were taken after 5, 10, 30 and 60 min and treated with 7 M of trichloroacetic acid. To determine the conditions required for the dehalogenase activity to be revealed, the cell-free extract was incubated under aerobic conditions, under oxygen or nitrogen at stirring at 30° C. NADH, NADPH and Fe^{2+} at a concentration of 0.1 mM were used as cofactors. Purity of $^{18}O_2$ was 81.7%, $H_2{}^{18}O$ – 81.05% both from v/o 'Izotop' USSR).

Table 1. Degradation of chlorophenols (%) by resting cells of *S. rochei* (inoculation time, 24 h).

Substrate	Concentration			
	10 ppm	20 ppm	50 ppm	100 ppm
3-CP	>90	>95	94.1	60
4-CP	73.6	0	0	0
2-CP	>90	>95	>98	>99
2,4-DCP	>90	>95	>98	>99
2,3-DCP	>90	>95	>98	56
2,5-DCP	>90	>95	>98	92
2,6-DCP	>90	>95	>98	>99
3,5-DCP	>90	>95	85.5	51.8
3,4-DCP	>90	>95	>98	93.8
2,4,6-TCP	>90	>95	>98	>99
2,4,5-TCP	>90	>95	63.6	30
2,3,4-TCP	>90	>95	>98	56
2,3,5-TCP	>90	>95	50	5
2,3,6-TCP	>90	>95	95	90
2,3,5,6-TeCP	>90	>95	70	20
PCP	>90	80	60	30
2,4-DBrP	>90	>95	>98	>99
2,6-DBrP	>90	>95	>98	>99

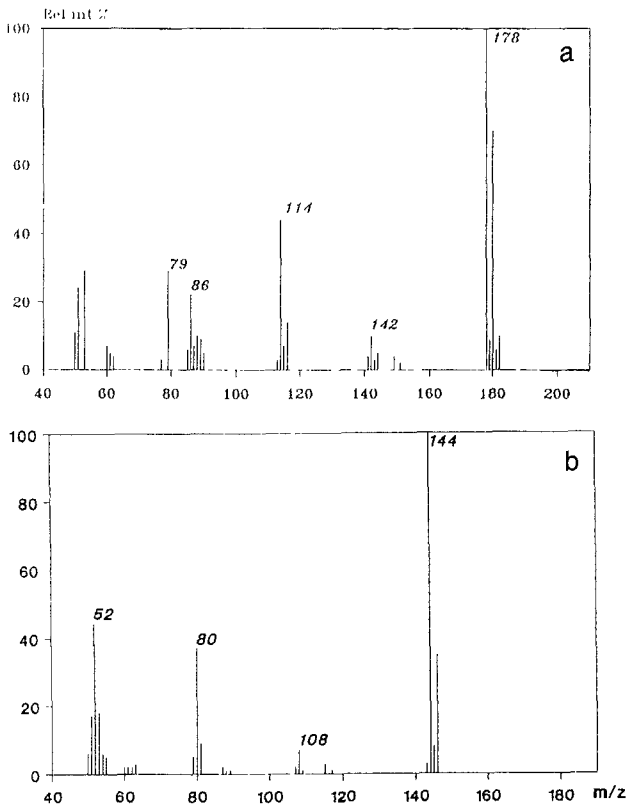

Fig. 4. Mass spectra of the intermediates of (a) degradation of 2,4,6-TCP and 2,6-DCP; (b) degradation of 2,4-DCP and 2-CP.

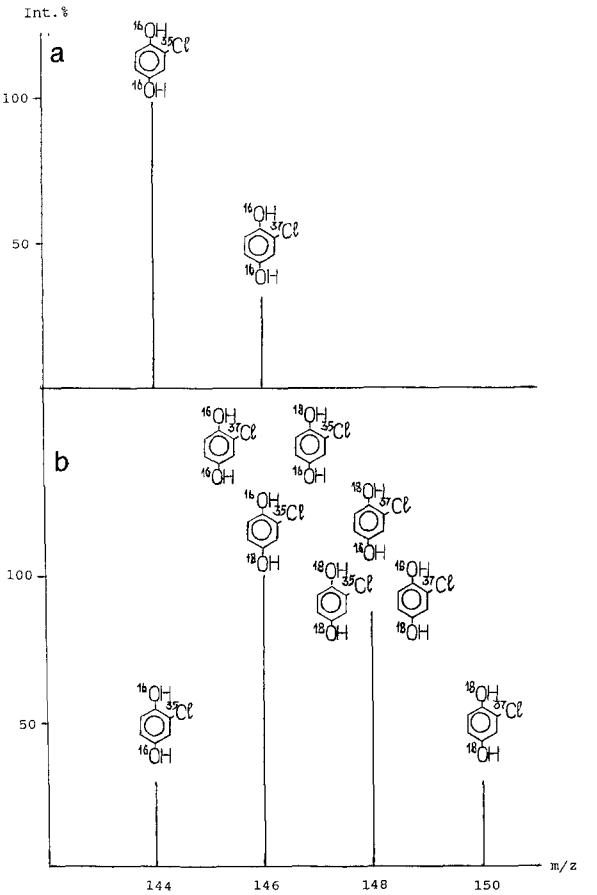

Fig. 5. Mass spectrometric analysis of the isotopic exchange of the nonenzymatic nature. (a) mass spectrum of chlorohydroquinol; (b) mass spectrum of chlorohydroquinol after 6 h of exposure to $H_2{}^{18}O$.

Ring cleavage enzyme assays

The enzymes of aromatic ring cleavage were determined in cell-free extracts obtained from the cells at the mid-exponential phase of growth on 2,4,6-TCP, 2,4- DCP, sodium succinate or sodium succinate with induction of 2,4,6-TCP, 2,4- and 2,6-DCP as described above.

Catechol 1,2-dioxygenase (Nakazawa & Nakazawa 1970; Dorn & Knackmuss 1980) and catechol 2,3-dioxygenase (Nozaki 1970) were measured spectrophotometrically. Hydroxyquinol 1,2-dioxygenase was determined by the changes in UV absorbance of HHQ during its incubation with the cell-free extracts: the novel peak ($\lambda = 243$ nm) appeared which vanished at acidification to pH 2.0. This is characteristic of maleyl acetate formation (Chapman & Ribbons 1976; Gaal & Neujahr 1979). A level of the hydroxyquinol 1,2-dioxygenase activity was established by the polarographic method described for a pure enzyme (Sze & Dagley 1984).

Solutions of HHQ and CHHQ in MES buffer were prepared under anaerobic conditions during the nitrogen blowing and then were added to the cell-free extract. The rates of O_2 consumption at the nonenzymatic oxidation of HHQ and CHHQ were subtracted from those during the oxidation of these compounds by the cell-free extract.

6-Chloro-2-hydroxyhydroquinol was synthesized from 3-chloro-4,5-dihydroxybenzaldehyde (Dakin 1909). Melting point, 135° C; IR spectrum (ν, cm^{-1}): 820, 1472, 1505, 1616 (aromatic ring); 3320 (OH). ^1H NMR (ppm) in d_6 acetone: 6.36 (d,

ArH), J 2.88 Hz; 6.39 (d, ArH), J 2.8 Hz; 7.32 (2 OH); 8.26 (OH) mass spectrum (Fig. 1).

Results and discussion

Chlorophenol utilization

The strain *Streptomyces rochei* 303 (VKM Ac-1284D) utilized 2-chloro-; 2,4-, 2,6-dichloro- and 2,4,6-trichlorophenols as the sole carbon source.

2,4,6-TCP was utilized preferentially (400 ppm, during 10–12 days) (Fig. 2). The degradation dynamics of 2,4-, 2,6-DCP and 2-CP were similar. The culture growth was shown to correlate with the chlorophenol uptake and chloride release into the medium. The lag period of some days is followed by an active culture growth and chlorophenol degradation. Changes in chlorophenol concentration affected the duration of the lag phase (Golovleva et al. 1991).

The *S. rochei* resting cells completely dechlorinated and degraded the chlorophenols under study (except 4-CP) at their concentration of 10 ppm in the medium (Table 1, Fig. 3). When the concentration of the toxicants was increased tenfold, they degraded completely 2-CP; 2,4-, 2,6-DCP; 2,4,6-TCP, 2,4-, 2,6-DBrP and partially converted all the isomeric mono- di-, trichlorophenols, tetrachlorophenol and pentachlorophenol. Only 4-CP (100 ppm) remained intact.

Pathways of chlorophenol degradation

We identified two intermediates formed during 2,4,6-TCP, 2,4-, 2,6-DCP and 2-CP degradation. One of the compounds was determined in the process of 2,4,6-TCP and 2,6-DCP decomposition. Based on the TLC, HPLC and GC/MS method and the data of its comparison with the standard it was identified as 2,6-dichlorohydroquinol (R_f, 0.52; R_t, 4.2 in system II; Fig. 4a). Another intermediate was detected during 2,4-DCP and 2-CP utilization and identified as chlorohydroquinol (R_f 0.45; R_t, 3.8 in system II; Fig. 4b). We failed to find any other intermediates in the process of chlorophenol degradation.

Dechlorination activity of the strain

The presence of oxygen and NADH proved necessary for the degradation of 2,4,6-TCP by the *S. rochei* 303 cell-free extract. Neither dechlorination nor degradation of the substrate was observed in the absence of the above factors. The highest dehalogenase activity was found in the presence of NADH under oxygen (Table 2). According to some data (Fetzner et al. 1989), Fe^{2+} is required for dechlorination. In our case, in the presence of Fe^{2+} the dehalogenase activity was lower.

The attempts to determine the nature of the dehalogenation enzyme did not produce the desirable result. Using $H_2{}^{18}O$, we showed that in the

Table 2. The dehalogenating activity of cell-free extract.

Reaction mixture	Dehalogenation activity, nmol of 2,4,6-TCP·min^{-1}·mg protein^{-1}
CFE, NADH, aerobic conditions	6.99
CFE, NADH, O_2	8.55
CFE, NADH, N_2	0.04
CFE, NADPH, O_2	1.6
CFE, NADH, Fe^{2+}, aerobic conditions	4.2
CFE, O_2	0.08

Amount of protein in the sample, 6 mg. CFE, cell-free extract.

Table 3. Activity of hydroxyhydroquinol 1,2-dioxygenase in the cell-free extract of *S. rochei* 303.

Growth substrate	Hydroxyhydroquinol 1,2-DO, nmol O_2·min^{-1}·mg protein^{-1}	
	Hydroxy-hydroquinol	6-Chloro-2-hydroxy-hydroquinol
2,4,6-TCP	14.1	43.7
2,4-DCP	22.0	14.6
Succinate + 2,4,6-TCP	2.84	24.4
Succinate + 2,4-DCP	6.88	7.26
Succinate + 2,6-DCP	4.78	6.75

control labelled oxygen incorporates into the chlorohydroquinol molecule already after 6 h owing to the isotopic exchange reactions of the nonenzymatic nature (Fig. 5). Such reactions proceed in conformity with the known properties of phenols (Oae et al. 1966) but the fact that for chlorohydroquinol they are realized in neutral solutions has not been described until now. Therefore, use of $H_2{}^{18}O$ does not make it possible to determine by involving which oxygen source chlorohydroquinol is formed.

We failed to detect any intermediates in experiments with $^{18}O_2$ because it strongly intensified the degradation.

We suggest that in this case the initial dehalogenation is performed by monooxygenase of the broad substrate specificity relative to halophenols which carries out dehalogenation in *para* position.

Enzymes of the aromatic ring cleavage

We searched for catabolic enzymes related to 2,4,6-TCP metabolism in a cell extract of the strain grown on 2,4,6-TCP. In addition to the dehalogenating enzyme, hydroxyquinol 1,2-dioxygenase was detected (Table 3). Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase were not detectable with catechol, indicating that 2,4,6-TCP is not metabolized through catechol. Trace activities of enzymes with 3- and 4-chlorocatechols were found. The enzymes detected in the cells grown on 2,4,6-TCP may be inducible, since none of these activities was detected in cells grown on *para*-hydroxybenzoate or succinate.

During the cell growth on succinate with the induction by various chlorophenols, the hydroxyquinol 1,2-dioxygenase activity was detected. Since we assumed that it would split the aromatic ring of incompletely dechlorinated hydroxyhydroquinol, we synthesized 6-chloro-2-hydroxy-hydroquinol and checked the possibility of its cleavage. As seen in Table 3, it is cleaved; if cells were grown on 2,4,6-TCP, the activity of the cell-free extract relative to 6CHHQ was higher than relative to nonchlorinated HHQ. The same regularity was observed during the induction of the succinate-grown cells by this phenol. In the case of the 2, 4-DCP, the activity of the cell-free extract was higher with unsubstituted HHQ and approximately the same with both substrates and the induction of 2,4-DCP.

To sum it up: we have isolated an *S. rochei* strain capable of growing within a wide range of chlorinated phenols, including 2-CP, 2,4-, 2,6-DCP; 2,4,6-TCP. Chlorophenol-degrading microorganisms are well reported in the literature. The corineform bacterium KC-3 utilizes 2.4,6-TCP for growth, yet is not active towards di- and monochlorophenols (Chu & Kirsch 1973); the strain *Flavobacterium* sp. utilizes PCP for growth only and degrades 2,4,6-TCP and 2,6-DCP solely under non-growth conditions (Steiert et al. 1987); *Rhodococcus* sp. CG1 and *Mycobacterium* sp. CG-2 degrade 2,4.6-TCP and 2,4-DCP only in the presence of glucose or mannose (Häggblom et al. 1988). As for the strain *S. rochei* 303, it utilizes isomeric chlorophenols as the sole carbon source. What is more, in contrast with the earlier isolated strains, it is capable of degrading both mono- and polychloro substituted phenols.

It was earlier reported that degradation of polychlorophenols can proceed via the formation of hydroxyquinol at the final stage of dechlorination (Apajalachti & Salkinoja-Salonen 1987b). Its further degradation can occur due to both the action of catechol 1,2-dioxygenase (Gall & Neujahr 1979) and hydroxyquinol 1.2-dioxygenase (Sze & Dagley 1984). Until now it has been common knowledge that the aromatic ring is cleaved by catechol 1,2-dioxygenase during degradation of chlorophenols by various microorganisms (Dorn & Knackmuss 1978; Gorlatov et al. 1989). We first revealed the involvement of hydroxyquinol 1,2-dioxygenase in the aromatic ring cleavage during the degradation of polychlorophenols. The data on the activity of HHQ 1.2-DO relative to nonsubstituted and monochlorosubstituted hydroxyhydroquionols suggest that, depending on cultivation conditions, two different enzymes of HHQ cleavage can be induced – type I and type II – by analogy with those for catechol 1,2-dioxygenase (Dorn & Knackmuss 1978). However, this suggestion stands in need of verification in further experiments.

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