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Degradation of 2,4,5-Trichlorophenoxyacetic acid by a *Nocardioides simplex* culture

Ludmila A. Golovleva, Raisa N. Pertsova, Ludmila I. Evtushenko & Boris P. Baskunov
Institute of Biochemistry and Physiology of Microorganisms, USSR Academy of Sciences, Pushchino, Moscow Region, 142292, USSR

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Abstract

A *Nocardioides simplex* strain 3E was isolated which totally dechlorinated 2,4,5-trichlorophenoxyacetic acid and was capable of its utilization as the sole source of carbon. The mechanism of 2,4,5-trichlorophenoxyacetic acid degradation by this strain was investigated. Chloroaromatic metabolites that occur in the lag, exponential and stationary growth phases of the strain *Nocardioides simplex* 3E were isolated and identified bases on a combination of TLC, GC-MS and HPLC data. Decomposition of 2,4,5-trichlorophenoxyacetic acid at the initial stage was shown to proceed by two pathways: via the splitting of the two-carbon fragment to yield 2,4,5-trichlorophenol and the reductive dechlorination to produce 2,4-dichlorophenoxyacetic acid. Hydrolytic dechlorination of 2,4,5-trichlorophenoxyacetic acid was found to yield dichlorohydroxyphenoxyacetic acid, thus pointing to the possible existence of a third branch at the initial stage of degradation of the xenobiotic. 2,4,5-Trichlorophenol and 2,4-dichlorophenoxyacetic acid produced during the metabolism of 2,4,5-trichlorophenoxyacetic acid and in experiments with resting cells are utilized by the strain *Nocardioides simplex* 3E as growth substrates.

Abbreviations: 2,4-D – 2,4-dichlorophenoxyacetic acid, 2,4,5-T – 2,4,5-trichlorophenoxyacetic acid, 2,4,5-TCP – 2,4,5-trichlorophenol

Introduction

Chloroaromatic componds used as herbicides, insecticides and wood preservatives accumulate in the environment due to their persistence. It is also known that microbial decomposition of these compounds in the biosphere may yield intermediates more hazardous for the atmosphere than the initial substances. Their metabolisms may lead to accumulation of polychlorophenols capable of condensing to polychlorodibenzodioxines that are extremely toxic and hazardous for the environment (Rochkind et al. 1986). Therefore, research into the metabolic pathways by active strains is of great interest.

2,4,5-T is one of the most persistent herbicides. As of today, no wild strain totally degrading this xenobiotic has been described. We have isolated the strain *Nocardioides simplex* 3E capable of utilizing 2,4,5-T as the sole source of cabon and energy at sufficiently high concentrations – up to 4.0 mM. This paper describes the strain and the pathway of 2,4,5-T metabolism by the active strain.

Materials and methods

Microorganism

The strain 3E totally utilizing 2,4,5-T was isolated from soil by the method of enrichment culture with 2,4,5-T as the sole source of carbon. Soils that had been under a long-term treatment by the pesticides were used as a source of microorganisms.

Identification of the strain

To study physiological and morphological characters and peculiarities of the life cycle of the strain, it was grown on BPA (beef extract agar) and tenfold-diluted BPA at 28° C. Cells were Gram-stained as described by Gregersen (1978). The composition of the cell wall was investigated using transmission microscopy.

When the strain was intended for biochemical analysis, it was grown in flasks with 100 ml of basic medium on a shaker (180 rpm) at 28° C up to the mid-logarithmic phase. Peptidoglycane was isolated and its composition established according to Schleifer & Kandler (1972). Amino acids were identified on an LC 6000 Amino Acid Analyzer (Biotroniks, FRG); an isomer of diaminopimelic acid was determined by paper chromatography (Lechevalier & Lechevalier 1980). The presence of mycolic acids was controlled by thin layer chromatography (Kroppenstedt & Kutzner 1981). Fatty acids were determined from their methyl esters obtained according to the technique of Farrow & Collins (1983), extracted with hexane and analyzed by gas-liquid chromatography on a Perkin-Elmer instrument (USA) equipped with an E-30 capillary column and a flame ionization detector. Menaquinones were extracted from dry cells and purified by thin layer chromatography (Collins 1985), their composition was determined using a Finnigan MAT 8430 mass-spectrometer (FRG).

DNA was isolated as described by Bradley et al. (1973). The GC-content was measured by the method of heat denaturation in 0.1 SSC (1 SSC = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0) using a Beckman DU-8B spectrophotometer with a thermostatted chamber and calculated from the formula proposed by Owen & Pitcher (1985). Physiological and biological features were studied using the methods of Goodfellow (1971) and Gordon et al. (1974).

Culture conditions

The strain was grown in a mineral chlorine-free medium containing (mM): KH_2PO_4, 0.7; $(NH_4)_2HPO_4$, 13.2; Na_2SO_4, 0.7; $MgSO_4 \cdot 7H_2O$, 1.2; KNO_3, 9.9; pH 7.0. The medium was sterilized at 120° C for 30 min, then supplemented with carbon sources 2,4,5-T (2.0 mM), 2,4-D (4.0 mM), 2,4,5-TCP (introduced portion-wise to a final concentration of 0.04 mM after 10–14 h).

The strain was cultured in shaken (180 rpm) flasks containing 200 ml medium at 29° C. Inoculum was cultured on a solid mineral (1.5% Difco) or liquid medium with 2,4,5-T (2.0 mM) for 72 h. Culture growth in the liquid medium was controlled spectrophotometrically.

Incubation with resting cells

The cells were grown in a mineral medium containing 2.0 mM of 2,4,5-T, centrifuged (10,000 *g*, 20 min at 4° C), washed twice with 50 mM Na-phosphate buffer (pH 7.0) and suspended in the same buffer. The suspension contained 1.0 g (wet weight) resting cells, 2.5 mM Na-phosphate buffer and 10 μM 2,4,5-T in a total volume of 50 ml. The reaction was carried out at 29° C on a shaker (180 rpm). Contents of mineral chlorine, 2,4,5-T, 2,4-D and 2,4,5-TCP were determined in samples taken 0.5, 1, 4, 6, 11 h after the incubation was initiated.

Isolation of metabolites

The cells were grown in flasks containing 200 ml medium with 2,4,5-T. The culture liquid supernatant was extracted three times with an equal volume of diethyl ether without or with acidifica-

tion with 0.1 N H_2SO_4 to pH 2.0. The extracts were collected and dried by dehydrated Na_2SO_4. The solvent was evaporated in a rotor evaporator and then under nitrogen flow to a volume of about 0.5 ml.

Analytical methods

The metabolites were identified by TLC, GC-MS and HPLC methods. TLC was done on Silufol UV-254 plates (Kavalier, Czechoslovakia) in two solvent systems. System A consisted of benzene : dioxane: acetic acid at a ratio of 90 : 10 : 2 ($v \cdot v^{-1}$); system B, of benzene : dioxane at a ratio of 90 : 10 ($v \cdot v^{-1}$). Chlorinated compounds were revealed using silver nitrate (Szokolay & Madaric 1969); phenolic substances were detected by diazobenzidine (Kirchner 1978).

Preparative isolation of the compounds was performed using system B. GC-MS was carried out at a 2091 instrument (LKB Instruments Inc., Bromma, Sweden). 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 three solvent systems: I, 5 mM KH_2PO_4 (adjusted to pH 2.0 with analytical-grade concentrated H_2SO_4): methanol 30: 70 ($v \cdot v^{-1}$); II, 5 mM KH_2PO_4 (adjusted to pH 2.0 with concentrated H_2SO_4): methanol 50: 50 ($v \cdot v^{-1}$); III, water: methanol 20 : 80 ($v \cdot v^{-1}$). The solvent flow rate was 0.7 ml $\cdot$ min^{-1}.

Quantitative determination of 2,4,5-T, 2,4-D and 2,4,5-TCP was performed in system I using the method of external standards. Concentration of chloride in the supernatant was determined by a chlorine-selective electrode on a EA 940 Ion Analyzer (Orion Research Inc., Cambridge MA, USA).

Results

Isolation of the active strain

Analysis of 2,4,5-T in enrichment cultures showed the total xenobiotic degradation only in the variant with 2,4,5-T added at a concentration of 0.04 mM.

The active enrichment culture capable of total 2,4,5-T degradation was obtained as the result of a 7-month cultivation (29 reinoculations) and was found to contain five microbial species including representatives of the *Pseudomonas* and *Micrococcus* genera, two *Bacillus* bacteria, and one unidentified coryneform bacterium. None of the microorganisms, isolated from this community as a pure culture, was able to grow on 2,4,5-T.

Subsequent cultivation (11 months, 32 passages) permitted selection of an association composed of two species (*Bacillus* sp. and a coryneform bacterium) and capable of mineralizing 2,4,5-T. Initially, none of the pure cultures showed growth on the xenobiotic; nonetheless, after a period of regular reinoculations (3 months, 19 passages) we succeeded in isolating from this microbial community a coryneform bacterium termed 3E which was capable of forming small colonies in the solid medium containing 2,4,5-T (0.04 mM) as the sole source of carbon.

Taxonomic characterization of the strain

When grown on a tenfold-diluted BPA, the strain 3E forms colonies up to 5 mm in diameter with a white or yellowish convex central region and a flat periphery. The edge of the colony is smooth or slightly wavy.

The development cycle of the strain is of a coccus-bacillus-coccus type. In the stationary growth phase, the culture is visualized as cocci of 0.5–0.6 μm in diameter and short rods with round ends which germinate upon their transfer on a fresh medium forming irregular motile rods 0.2–0.3 μm long, characteristic of the culture in the exponential growth phase (Fig. 1).

Cells of one-day culture become Gram-positive. Electron microscopy of the cell wall structure re-

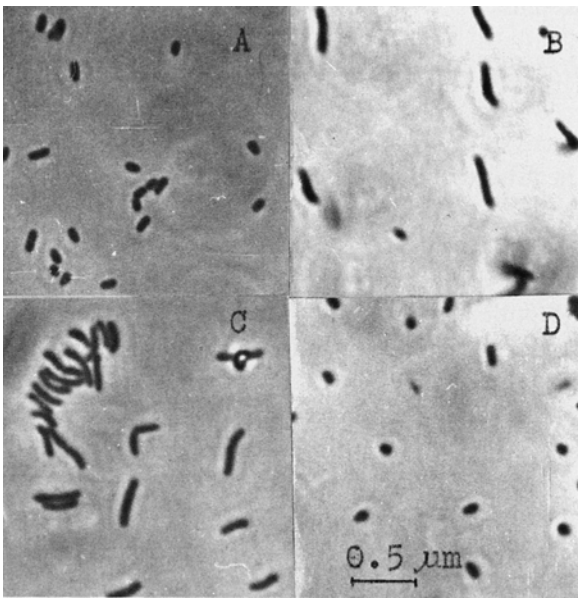


Fig. 1. Morphological cycle of *Nocardioides simplex* 3E grown on BPA at 29° C for 0 h (A), 7 h (B), 24 h (C), 7 days (D).

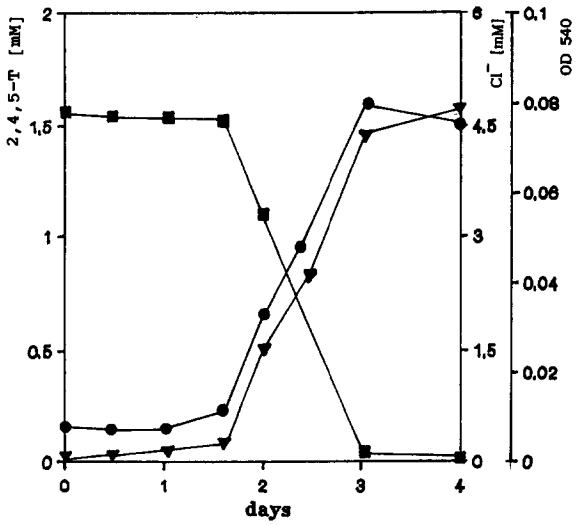


Fig. 2. Dynamics of 2,4,5-T transformation by *Nocardioides simplex* 3E. ■, 2,4,5-T content; ▲, Cl^- release; ●, optical density.

vealed two electron-dense rigid layers. Certain cross sections showed a diffuse porous amorphous layer, apparently, of polysaccharide origin. The outer membrane layer is absent.

The peptidoglycane was found to contain LL-diaminopimelic acid, glycine and alanine, glutamic acid, *N*-acetylmuramic acid and glucosoamine. Mycolic acids were not found. As regards fatty acids, predominant are iso-, anteisobranched ($iC_{16:0}$; $iC_{18:0}$; $aC_{17:0}$), nonbranched mononsaturated ($C_{16:1}$; $C_{18:1}$) acids; nonbranched saturated ($C_{16:0}$-$C_{18:0}$) and 10-methyl-branched acids, including tuberculostearic acid, and finally, two hydroxy acids were also detected.

As the basic quinone we revealed saturated menaquinone with eight isoprenoid units MK 8(H4). The strain was found:

- to be aerobic;
- to give positive catalase and negative oxidase reactions;
- to utilize glucose, trehalose, sucrose, fructose as the sole source of carbon, converting them into acids;
- not to transform into acids adonite, *L*-arabinose, inositol, xylose, mannitol, maleose, maltose, *L*-ramnose, raffinose, ribose, cellobiose;
- to utilize acetate, pyruvate, succinate, malate, citrate, fumarate, α-ketoglutarate, formiate, malonate, *p*-hydroxybenzoate, protocatechoate, gentisate, homogentisate, homoprotechoate;
- not to utilize benzoate, tartrate, oxalate, valeriate, salicylate, phenoxyacetate;
- to hydrolyze starch, caseine;
- not to hydrolyze arbutine;
- to display nitrate reductase activity.

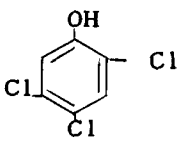
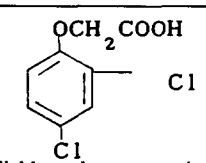
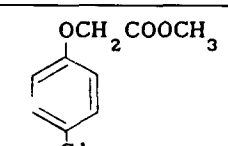
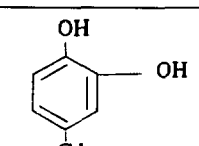
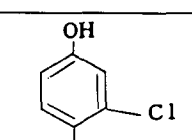
The strain grows in the temperature range of 17–37° C, the optimal growth temperatures are 28–30° C. By its morphological, physiological and hemotaxonomic characters, the strain is very close to the *Pimelobacter simplex* (Suzuki & Komagata 1983). Bearing in mind that the latter has recently been attributed to the *Nocardioides* genus (Collins et al. 1989), our strain was classified as *Nocardioides simplex*.

Utilization of 2,4,5-T by the strain 3E

Investigation of the strain growth dynamics in a medium with a gradient of 2,4,5-T concentrations (0.8–4.0 mM) indicates that the lag-period of the first two days is followed by an active culture growth during which the growth duration changes insignificantly. The culture growth shows a clear-cut correlation with the 2,4,5-T uptake and chloride release into the medium (Fig. 2), the most active 2,4,5-T utilization and release of chloride coinciding with the beginning of exponential growth. Upon total depletion of 2,4,5-T the stoichiometric quantity of mineral chloride is determined in the medium.

Using the methods of TLC, GC-MS and HPLC (Table 1) we have isolated and identified 5 metabolites of 2,4,5-T. These are a trichloro-containing intermediate 2,4,5-trichlorophenol; a dichloro-containing metabolite 2,4-dichlorophenoxyacetic acid; and monochlorinated compounds 4-chlorophenoxyacetic acid, 4-chlorocatechol and chloro-

Table 1. Characteristics of products of 2,4,5-T metabolism by the strain *N. simplex* 3E.

Metabolite	Major intensive peaks in the mass spectrum (%)		R_t in system II (HPLC)	R_f in system B (TLC)	Established structure
M_1	200(31)	198(98)	12.96	0.72	 2,4,5-trichlorophenol
	196(100)M^+	135(10)			
	134(20)	133(14)			
	132(31)	99(20)			
	98(9)	97(45)			
M_2	236(54)	234(82)M^+	6.68	0.66	 dichlorophenoxyacetic acid
	201(32)	199(96)			
	177(65)	175(100)			
	163(34)	161(49)			
	147(41)	145(48)			
M_3	202(25)	200(75)M^+	4.99	0.58	 methyl ester of 4-chlorophenoxyacetic acid
	143(35)	141(100)			
	113(32)	111(95)			
	101(5)	99(15)			
	76(33)				
M_5	146(34)	144(100)M^+	3.98	0.42	 4-chlorocatechol
	126(9)	115(8)			
	100(9)	98(27)			
	81(15)	63(33)			
	51(18)				
M_6	146(34)	144(100)M^+	3.21	0.50	 chlorohydroquinone
	108(7)	81(9)			
	80(37)	79(5)			
	53(18)	52(44)			
	51(17)				

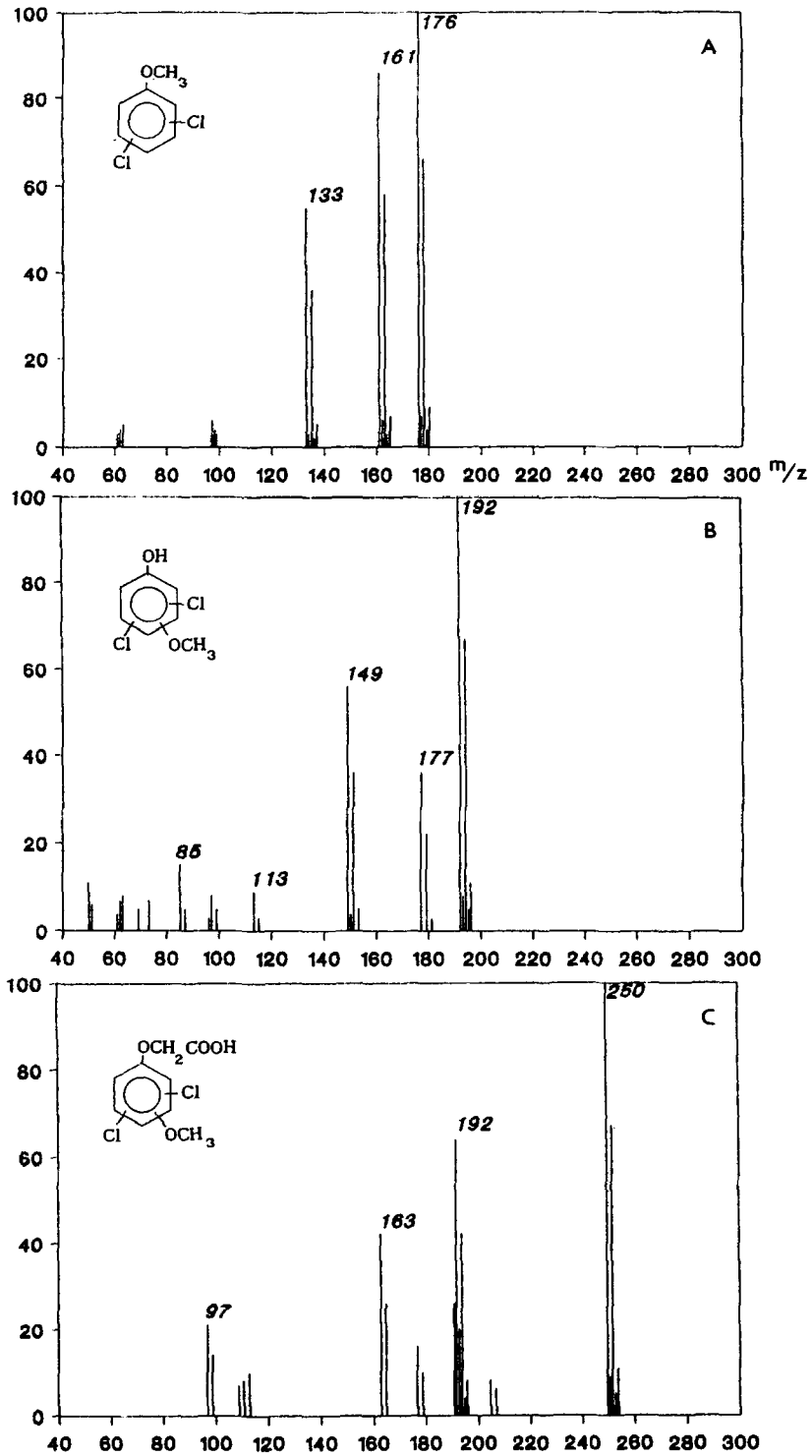


Fig. 3. Mass spectra of products of 2,4,5-T degradation by *Nocardioides simplex*3E. (A) dichloro-*o*-methylphenol; (B) dichloromethox yphenol; (C) dichloromethoxyphenoxyacetic acid.

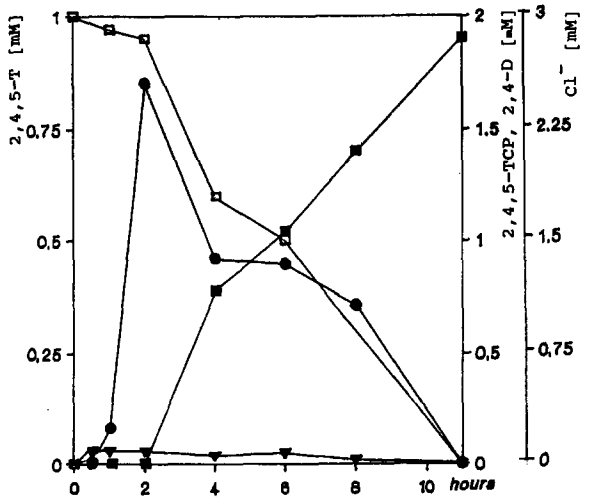


Fig. 4. Dynamics of 2,4,5-T conversion by *Nocardioides simplex* 3E resting cells. □, 2,4,5-T; ● 2,4-D; ▲ 2,4-TCP; ■, Cl^-.

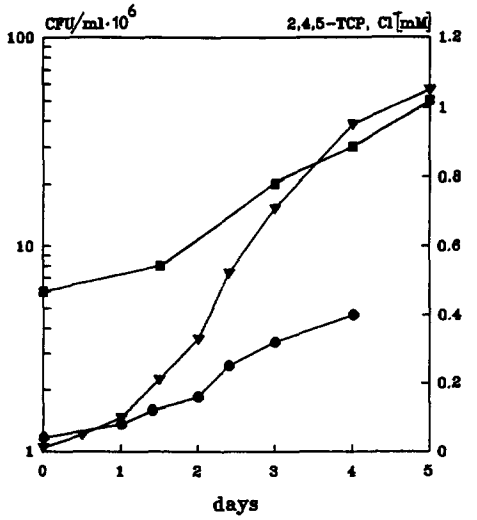


Fig. 5. Dechlorination of 2,4,5-TCP by the strain *Nocardioides simplex* 3E. ●, total 2,4,5-TCP added; ▲ Cl^- release; ■, viable cells.

hydroquinone. We also present the mass-spectrometry data on the 2,4,5-T methoxylated decomposition products dichloro-*O*-methylphenol, dichloromethoxyphenol and dichloromethoxy-phenoxyacetic acid (Fig. 3).

In the lag phase (1–2 days) only 2,4,5-trichlorophenol was found. The exponential phase (3–4 days), along with 2,4,5-TCP, yielded 2,4-D. These two metabolites can be considered as products of the first stage of 2,4,5-T degradation by the strain 3E. Besides 2,4,5-TCP and 2,4-D, the exponential phase also revealed the products of more intensive decomposition – 4-chlorophenoxyacetic acid, 4-chlorocatechol, chlorohydroquinone, and the methoxyderivatives of dichlorophenoxyacetic acid and dichlorophenol. At the stationary growth phase, 2,4,5-TCP and 2,4-D were not found, the compounds detected were only the dichlorocatechol and dichlorophenol methoxyderivatives.

Mineral chloride liberated as a result of 2,4,5-T dechlorination appeared in 2 days, in the exponential growth phase, the release of chlorine being complete in 4 days. Its content was equimolar to the amount of 2,4,5-T introduced.

Degradation of 2,4,5-T by resting cells

The dynamics of 2,4,5-T degradation by the strain 3E was studied in a suspension of resting cells (Fig. 4). The products determined were 2,4,5-TCP and 2,4-D which, as mentioned above, can be considered as those of the initial stages of 2,4,5-T degradation, 2,4,5-TCP occurred in the culture medium already in 30 min from the beginning of the incubation; 2,4-D was found after about 1 h. Interestingly, under growth conditions 2,4-D also appeared later than 2,4,5-TCP. Affter 11 h from the beginning of the incubation 2,4,5-T and its metabolites vanished from the medium and an equimolar amount of chloride appeared.

Degradation of 2,4-D and 2,4,5-TCP by strain 3E

To elucidate the capability of the strain 3E to utilize 2,4,5-TCP and 2,4-D and determine their maximal concentrations utilized by the active strain, we did a series of experiments with these compounds as growth substrates. It was shown that 2,4-D and 2,4,5-TCP could be consumed by the culture as sole carbon and energy sources. The strain 3E grew on 2,4-D at a concentration up to 8.0 mM, which was twice as much as the maximal concentration of

2,4,5-T utilized by this strain. The growth was characterized by a pronounced relationship between the disappearance of the substrate, release of chlorine and increment of biomass.

In contrast with 2,4-D, concentrations of 2,4,5-TCP were minor (0.04 mM). Multiple portion-wise additions of 2,4,5-TCP at a lower-than-maximal concentration and 10–14 h intervals sufficient for its total degradation caused the occurrence of stoichiometric amounts of chloride in the medium and culture growth (Fig. 5). Higher concentrations of 2,4,5-TCP were toxic for the culture. Thus, introduced at a concentration of 0.08 mM, it was not consumed by the culture and not degraded. The same concentration of 2,4,5-TCP, added to the culture grown on 2,4,5-T, completely arrested the bacterial growth.

Discussion

Abundance of metabolites of 2,4,5-T degradation by strain 3E indicates that the decomposition protocol is complex. Based on the data obtained, we could propose the scheme of initial metabolic stages of the xenobiotic. Growth of the culture on 2,4,5-T yielded 2,4,5-TCP, 2,4-D and methoxy-2,4-D. These compounds can be assumed to be the products of the first stage of 2,4,5-T metabolism.

Formation of 2,4,5-TCP as a result of 2,4,5-T two-carbon fragment splitting is characteristic of all known cultures transforming 2,4,5-T (Rochkind et al. 1986; Rosberg & Alexander 1980). However, 2,4,5-TCP is, as a rule, the end product of 2,4,5-T degradation by microorganisms. Strain 3E is able to mineralize this compound completely, as another known strain utilizing 2,4,5-T, *Pseudomonas cepacia* AC 1100 (Karns et al. 1983).

Formation of 2,4-D as a result of reductive dechlorination of 2,4,5-T under aerobic conditions has not been described in the literature. However, for other chlorinated compounds this reaction is known. Thus, recent works have described the processes of reductive dechlorination under aerobic conditions for the culture *Alcaligenes denitrificans* NTB-1 that converts 2,4-dichlorobenzoate to 4-chlorobenzoate (van den Tweel et al. 1987) and the strain *Rhodococcus chlorophenolicus* that dechlorinates pentachlorophenol to 1,2,4-trihydroxybenzene (Apajalachti & Salkinoja-Salonen 1987).

It is probable that, besides reductive dechlorination, the 2,4,5-T can be also metabolized via hydrolytic dechlorination producing hydroxydichloro-phenoxyacetic acid, because a methoxyderivative of dichlorophenoxy acid was found during the growth of the culture on 2,4,5-T. Apparently, a similar pathway also exists for the strain *P. cepacia* AC 1100 because the same metabolite has been found for this strain as well (Chakrabarty 1987).

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