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SHORT COMMUNICATIONS

Transformation of 3,4-Dichloroaniline under Conditions Promoting Nitrate Reduction

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Chloroanilines are toxic and persistent compounds presenting a real ecological hazard. Their occurrence in the environment is mainly due to the aerobic biotic transformation of herbicides, phenylurea, phenylcarbamates, and acylamides. Under anoxic conditions, aniline derivatives may result from the microbial reduction of nitrobenzene [1].

The first evidence for the microorganism capable of utilizing 3,4-dichloroaniline was obtained by Surovtseva *et al.* [2], who described the *Pseudomonas diminuta* strain, which dehalogenated 3-chloro-, 4-chloro-, and 3,4-dichloroanilines and utilized these compounds as the sources of carbon, nitrogen, and energy, but was unable to utilize unsubstituted aniline. Later, Surovtseva *et al.* [3] isolated the *Alcaligenes* sp. strain that was able to utilize not only 3-chloro-, 4-chloro-, and 3,4-dichloroaniline, but also unsubstituted aniline. Both strains utilized these xenobiotics under aerobic conditions via the modified *ortho*-pathway with the formation of (chloro)pyrocatechols.

The microbial degradation of xenobiotics under anoxic and slightly oxic conditions was studied only in regards to unsubstituted aniline. For instance, Schnell *et al.* [4] isolated and described the sulfate-reducing bacterium *Desulfobacterium anilini* Anil (DSM 4660) which utilized aniline and dihydroxybenzenes under anaerobic conditions. To the best of our knowledge, there have not yet been reports on the anaerobic microbial degradation of chlorosubstituted anilines.

For this reason, we undertook investigations to elucidate the possibility of the degradation of 3,4-dichloroaniline by enrichment cultures under conditions promoting nitrate reduction. Samples of sewage sludge were taken from the settlement pond of a petroleum bulk plant in Serpukhov (Moscow oblast). To obtain enrichment cultures, the samples in an amount of 30 g of wet biomass were placed in 300-ml plastic flasks with screw sealing closures and poured with a medium of the following composition (g/l): Na_2HPO_4, 2.2; KH_2PO_4, 0.16; $MgSO_4$, 0.1; $NaHCO_3$, 2.0; K_2HPO_4, 0.42; $(NH_4)_2SO_4$, 1.0; NaH_2PO_4, 1.2; and KNO_3, 2.0. The medium also contained selenium and tungsten salts [5]. A mixture of glucose, glycerol, calcium lactate, and sodium succinate (0.5 g/l of each) was used as a cosubstrate. Cysteine hydrochloride and sodium sulfide were added as reducing agents [6]. To remove oxygen from the medium, it was heated and then purged with nitrogen [6]. 3,4-Dichloroaniline was added in an amount of 100 mg/l, dissolving it in 1 ml of dimethylformamide. Enrichment cultures were reinoculated every 25 days. The incubation temperature was 36°C. Sterile medium was used as the control.

To quantify 3,4-dichloroaniline, 20 ml of the culture liquid was extracted thrice with ethylacetate. The pooled extract was evaporated and analyzed by high-performance reversed-phase chromatography on a 2134 LKB prepacked column (4.0 × 250 mm; Spherisorb ODS2; 5 μm particle size). The eluting solvent was a mixture of 5 mM KH_2PO_4 (pH 2.0) and methanol (30 : 70, v/v). The eluate was detected at 290 nm.

Decomposition products were identified by mass spectrometry on a Finnigan MAT model 8430 mass spectrometer (Germany) with the direct injection of samples to the region of ionization under standard conditions.

After 6 months of adaptation, the concentration of 3,4-dichloroaniline in the enrichment culture showed a tendency to diminish: over a period of 20–25 days, the amount of the toxicant decreased by 15–20%. The major reaction product identified in the culture liquid was the dimer of the parent molecule with a molecular mass of 333 (see table).

After 12 months of adaptation, 3,4-dichloroaniline was transformed more intensively by the enrichment culture, so that the xenobiotic was almost completely degraded over a 25-day period (Fig. 1). In this case, the reaction products were present in the culture liquid only in trace amounts (Fig. 2).

The data of mass spectrometry allowed us to determine the putative structure of some products of the 3,4-dichloroaniline transformation (see table). The accumulation of the compounds denoted as 6, 7, and 8 indicated that the bioconversion of 3,4-dichloroaniline involves the reactions of deamination, dechlorination, and carboxylation. Carboxylation is one of the key

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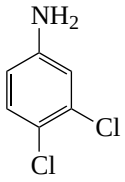
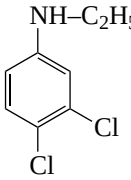
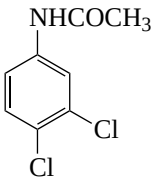
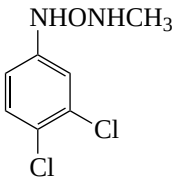
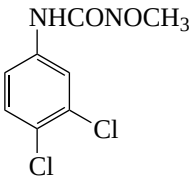
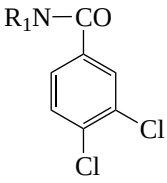
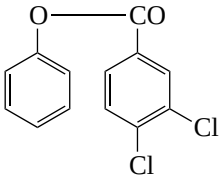
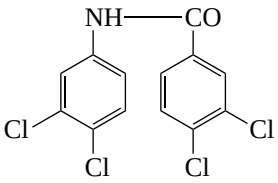
Mass spectra of the products of the 3,4-dichloroaniline transformation by the adapted enrichment culture

No.	*m/z* and relative abundance (%) of major peaks	Putative structure
1	M^+161(100), 163(65), 134(5), 126(13), 128(4), 99(22), 101(8), 90(21), 63(20)	
2	M^+189(56), 191(33), 161(100), 163(64), 125(18), 126(17), 127(7), 128(6), 90(19)	
3	M^+203(21), 205(14), 161(100), 163(65), 133(4), 126(7), 99(7), 90(6)	
4	M^+206(4), 208(3), 160(100), 162(66), 133(43), 135(27), 125(30), 127(11)	
5	M^+248(21), 250(14), 187(3), 188(3), 189(2), 160(14), 162(10), 125(12), 61(100)	
6	M^+207(4), 209(3), 179(10), 181(8), 173(38), 175(25), 145(100), 147(64), 109(32), 111(23)	
7	M^+266(34), 268(22), 145(20), 147(14), 121(68), 93(100)	
8	M^+333(4), 335(5), 337(2), 173(44), 175(26), 145(100), 147(65), 109(21), 111(7), 74(7), 75(7)	

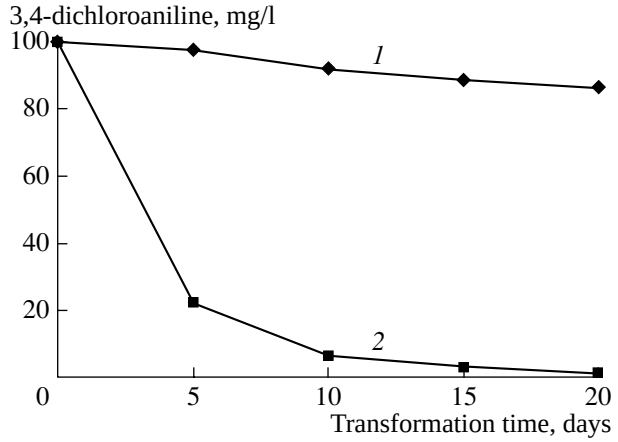


Fig. 1. Dynamics of the 3,4-dichloroaniline transformation by the enrichment culture adapted for (*1*) 6 months and (*2*) 12 months.

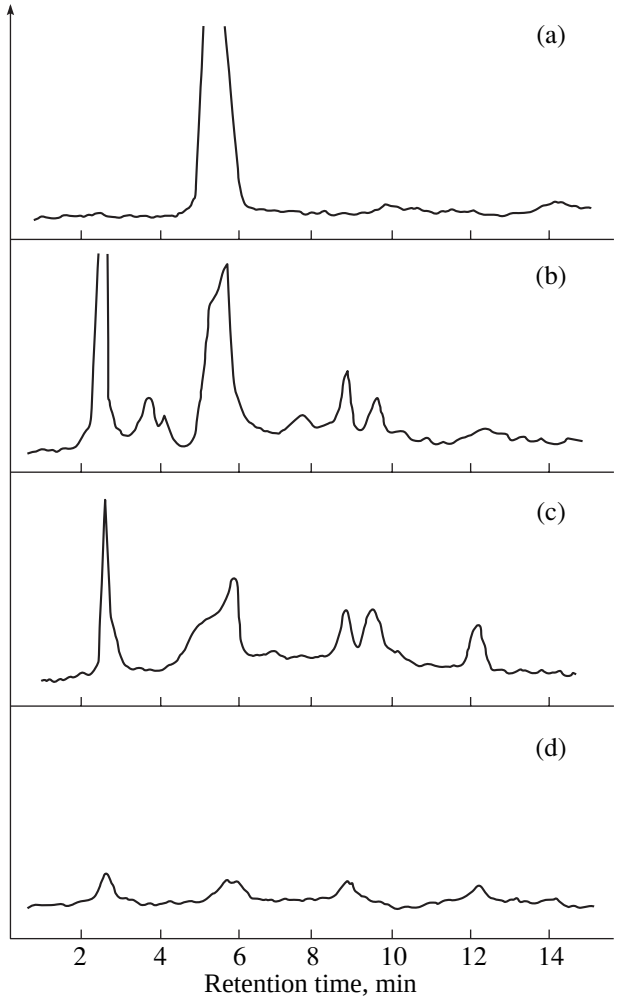


Fig. 2. Chromatography of the products of the 3,4-dichloroaniline transformation by the adapted enrichment culture after (a) 0, (b) 5, (c) 10, and (d) 20 days of incubation. The retention time of 3,4-dichloroaniline is 5.25 min.

reactions in the anaerobic peripheral metabolism of a wide range of aromatic compounds [7]. The deamination of the aromatic ring of 3,4-dichloroaniline allows the culture to use this substrate as the sole source of nitrogen, although the xenobiotic was transformed at a much lower rate if the cultivation medium did not contain an additional source of nitrogen. It should be noted that the analysis of the cultivation medium showed the absence of the typical compounds of the anaerobic transformation of 3,4-dichloroaniline, namely, unsubstituted or chlorinated benzoate.

The inoculation of nutrient agar with the 1-day-old enrichment culture led to the formation of colonies of six morphotypes. The dominant morphotype was represented by glossy leather-colored colonies, turning cream-colored with aging, irregular, 3–5 mm in diameter, with a conic profile, and a undulate edge. The bacterial strain was gram-positive and polymorphic: the 12-hour culture was represented by cocci, while the 24-hour culture, by rods. Using the identification criteria of *Bergey's Manual* [8], the isolated culture was preliminarily assigned to the genus *Rhodococcus.*

Like the adapted enrichment culture, the pure culture was found to completely degrade 3,4-dichloroaniline, though over a more extended cultivation period (35–40 days). In this case, the chromatographic pattern of the conversion products of 3,4-dichloroaniline was close to that observed in the case of the adapted enrichment culture.

Thus, we showed that 3,4-dichloroaniline can be degraded by the adapted enrichment culture and by the dominant microorganism isolated from this culture. Relevant investigations concerned with the conclusive identification of these microorganisms and the determination of the 3,4-dichloroaniline transformation pathway are in progress in our laboratory.

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