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SHORT COMMUNICATIONS

The Degradation of 3,4-Dichloroaniline by *Pseudomonas fluorescens* Strain 26-K

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Anilines and chloroanilines are widely spread hazardous pollutants of the environment, into which they are discharged as by-products of the industrial production of polyurethane, azodyes, rubbers, lacquers, and herbicides or as products of the microbial degradation of acylanilides, phenylurea, and phenylcarbamates [1–3]. There is an abundant literature on microbial strains capable of degrading unsubstituted aniline [1, 4–6], or utilizing monochloroanilines as sole sources of carbon and energy [7–9], or degrading them cometabolically [10]. At the same time, there is little information concerning the biodegradation of 3,4-dichloroaniline, which is very recalcitrant to microbial attack. Some of the first researchers to report on the microbial metabolism of 3,4-dichloroaniline were Zeyer and Kearney, who described the *Pseudomonas* sp. strain G that was able to degrade 3,4-dichloroaniline in the presence of 4-chloroaniline or succinate and ammonium nitrate as additional substrates [11], and In-Soon and Bartha, who described the *Pseudomonas putida* strain that was able to degrade 3,4-dichloroaniline in the presence of unsubstituted aniline or propioanilide [12].

Surovtseva *et al.* were the first to report on the ability of microorganisms to utilize not only 3-chloroaniline and 4-chloroaniline but also 3,4-dichloroaniline as the growth substrates [13]. The *Pseudomonas diminuta* strain described in this publication was able to utilize 3,4-dichloroaniline at concentrations of up to 50 mg/l. Later, Surovtseva *et al.* described the *Alcaligenes* sp. strain that was able to degrade the same substrates as the *P. diminuta* strain [14] and showed that 3,4-dichloroaniline-degrading strains can be used for the bioremediation of polluted soils [15].

In this paper, we report on the ability of *Pseudomonas fluorescens* strain 26-K, which was isolated from sewage of the coking industry [16], to utilize 3,4-dichloroaniline as the sole source of carbon, nitrogen, and energy.

P. fluorescens strain 26-K was grown at 36°C in shaken (180 rpm) Erlenmeyer flasks with 200 ml of a liquid mineral medium containing (g/l) Na_2HPO_4, 0.73; KH_2PO_4, 0.5; $MgSO_4$, 0.2; $NaHCO_3$, 0.25; $MnSO_4$, 0.001; NH_4NO_3, 0.75; and $FeCl_3$, 0.02. In some experiments, the strain was grown in the medium without ammonium nitrate. Glucose as a cosubstrate was added at a concentration of 1 g/l.

3,4-Dichloroaniline was added to the medium in the form of an acetone solution to concentrations from 25 to 250 mg/l. This compound was quantitatively analyzed using a Waters HPLC system equipped with a (4×250 mm) reversed-phase column packed with Spherisorb ODS2 (average particle size 5 μm). The column was eluted with a linear gradient of 1% acetic acid (5–95%) in methanol at a flow rate of 1 ml/min (total elution time 20 min). The absorption of eluted 3,4-dichloroaniline was recorded at 250 nm using a 2487 detector. 3,4-Dichloroaniline was identified by HPLC with the authentic sample of this compound and by mass spectrometry using a Finnigan MAT INCOS 50 mass spectrometer with an electron impact energy of 70 eV.

To obtain cell-free extract, washed cells were resuspended in a Tris–HCl buffer (pH 7.2) and disrupted in a press at a pressure of 3200 kg/cm^2. The cell homogenate was treated with DNase and then centrifuged at 17000 *g* for 30 min to remove cell debris. The supernatant was used for enzyme assay, which was carried out spectrophotometrically using a Shimadzu UV-160 spectrophotometer. Hydroxyquinol 1,2-dioxygenase was assayed by measuring the absorption of maleylacetate at 243 nm [17]. Protocatechuate 3,4-dioxygenase was assayed from the decrease in absorption at 290 nm [18]. Catechol 1,2-dioxygenase was assayed by measuring absorption at 260 nm [19]. Catechol 2,3-dioxygenase was assayed from the increase in absorption at 375 nm [20].

Figure 1 illustrates the growth of *P. fluorescens* 26-K on 3,4-dichloroaniline and its degradation under different conditions. In the presence of an additional carbon source, cells began growing virtually without lag-phase. In the media without a nitrogen source, or without an additional carbon source, or without a carbon cosubstrate, the lag-phase was extended and the growth rate was relatively low, the doubling time remaining, however, at a level of no less than 1 day.

The analysis of the culture liquid of the *P. fluorescens* strain 26-K grown on 3,4-dichloroaniline in the presence of glucose (Fig. 2) showed that the concentra-

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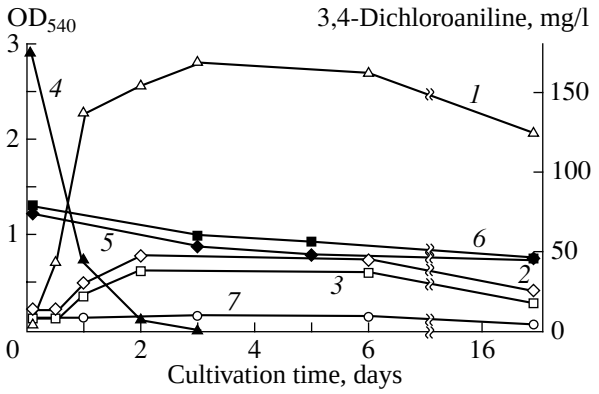

Fig. 1. Growth of *P. fluorescens* 26-K and the dynamics of 3,4-dichloroaniline degradation. Curves: (*1*) growth in the presence of glucose; (*2*) growth in the absence of glucose; (*3*) growth in the absence of glucose and nitrogen source; (*4–6*) decline in the concentration of 3,4-dichloroaniline in the culture liquids of variants (*1–3*); (*7*) control growth in the absence of glucose, nitrogen source, and 3,4-dichloroaniline.

tion of this compound decreased by 75% after 1 day of cultivation. On the following day, 3,4-dichloroaniline was detected in only trace amounts. After 5 days of cultivation, 3,4-dichloroaniline and its conversion products were almost entirely absent from the medium. In the control (uninoculated) medium, the concentration of added 3,4-dichloroaniline did not change throughout the cultivation period.

As can be seen from the table, in the medium without glucose, 3,4-dichloroaniline added at concentrations of 75, 25, and 15 mg/l was degraded by 40, 88, and 100% after 17 days of cultivation. In the medium without glucose and a nitrogen source, 3,4-dichloroaniline was degraded to almost the same extent (by 40, 84, and 100%, respectively). When the initial concentration of 3,4-dichloroaniline was 15 mg/l, the intermediate products of its metabolism were not detected in the culture liquid. In the medium with glucose as a cosubstrate, 3,4-dichloroaniline, even when it was added at a high concentration (250 mg/l), was entirely degraded over 4 days. In this case, the intermediate products of 3,4-dichloroaniline metabolism could not be detected in the culture liquid after 5–7 days of cultivation.

The measurement of the activities of enzymes involved in the cleavage of the aromatic ring of 3,4-dichloroaniline (pyrocatechase, metapyrocatechase, hydroxyquinol 1,2-dioxygenase, and protocatechuate 3,4-dioxygenase) in the extract of cells grown on this compound in the presence of glucose revealed the presence of very active metapyrocatechase (230 μmol/(min mg protein)), whereas the other enzymes were not detected. This finding suggests that the aromatic ring of 3,4-dichloroaniline is cleaved in the *meta* position. The question of the dechlorination and desamination of 3,4-dichloroaniline remains to be

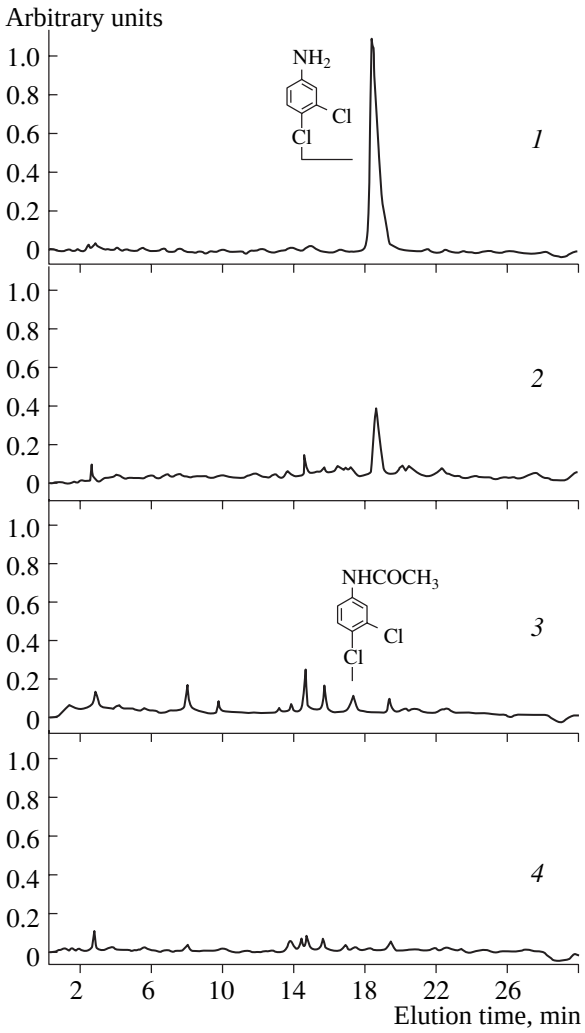

Fig. 2. The reversed-phase HPLC spectra of the culture liquid of *P. fluorescens* 26-K grown in the medium with glucose for (*1*) 0, (*2*) 1, (*3*) 3, and (*4*) 5 days. The initial concentration of 3,4-dichloroaniline in the medium was 170 mg/l.

answered. It should be noted that 3,4-dichloroacetanilide, which was detected in the culture liquid in trace amounts and disappeared by the end of the cultivation period (Fig. 2), is presumably a by-product of 3,4-dichloroaniline metabolism. The data presented indicate that *P. fluorescens* strain 26-K, which was isolated from sewage of the coking industry, is able to degrade relatively large amounts of 3,4-dichloroaniline.

In order to be used for the bioremediation of soils and waters polluted with a toxicant, degrading strains must be tolerant of this toxicant [15]. For instance, *Delftia* sp. AN3 can utilize unsubstituted aniline when its concentration is as high as 5 g/l [6]. At the same time, the recently described 3,4-dichloroaniline degraders show a low level of tolerance to this toxicant. For instance, the degradation of 3,4-dichloroaniline by *Pseudomonas* sp. strain G was inhibited by concentra-

Degradation of 3,4-dichloroaniline by *P. fluorescens* strain 26-K

Medium	Concentration of 3,4-dichloroaniline (mg/l) in the culture liquid after the indicated number of cultivation days					
	0	1	2	3	5	17
With glucose	250	150	80	7	0	0
	170	45	3	0	0	0
Without glucose	75	68	–	55	50	45
	25	21	–	17	15	3
	15	12	–	10	5	0
Without glucose and nitrogen source	75	70	–	65	53	45
	25	23	–	18	15	4
	15	12	–	11	5	0

Note: The dash stands for “no data available.”

tions higher than 16 mg/l [11]. *P. putida* and *P. diminuta* were able to degrade 3,4-dichloroaniline at concentrations equal to 2 and 50 mg/l, respectively [12, 13]. The highest tolerance of bacteria to 3,4-dichloroaniline reported so far (up to 150 mg/l) was that of *Paracoccus denitrificans* [8].

In this communication, we reported on the ability of *P. fluorescens* strain 26-K to utilize 3,4-dichloroaniline either as the sole source of carbon, nitrogen, and energy or cometabolically. In the latter case, a concentration of 3,4-dichloroaniline as high as 250 mg/l does not inhibit the degradation rate of this toxicant, which indicates the potential for using *P. fluorescens* strain 26-K for bioremediation purposes.

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