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Exploited Application of a Newly Isolated *Pseudomonas acidovorans* XII in Microbial Degradation of 1-Chloro-4-Nitrobenzene

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Abstract Bacterial strain XII, which belongs to the family Pseudomonad, utilizes 1-chloro-4-nitrobenzene as a sole source of carbon, nitrogen, and energy. Suspensions of 1-chloro-4-nitrobenzene -grown cells removed 1-chloro-4-nitrobenzene from culture fluids, and there was a concomitant release of ammonia and chloride. Under anaerobic conditions XII transformed 1-chloro-4-nitrobenzene into a product which was identified as 2-amino-5-chlorophenol by 1H and 13C nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. This transformation indicated that there was partial reduction of the nitro group to the hydroxylamino substituent, followed by Bamberger rearrangement. In the presence of oxygen but in the absence of NAD, fast transformation of 2-amino-5-chlorophenol into a transiently stable yellow product was observed with resting cells and cell extracts. This compound exhibited an absorption maximum at 395 nm and was further converted to a dead-end product with maxima at 226 and 272 nm. The compound formed was subsequently identified by 1H and 13C NMR spectroscopy and mass spectrometry as 5-chloropicolinic acid. In contrast, when NAD was added in the presence of oxygen, only minor amounts of 5-chloropicolinic acid were formed, and a new product, which exhibited an absorption maximum at 306 nm, accumulated.

Keywords: *Pseudomonas acidovorans, microbial degradation, NMR, 1-chloro-4-nitrobenzene*

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1. Introduction

Microbial bioremediation can be an efficient, economic and environmentally friendly alternative to other physico-chemical approaches used for the cleanup of contaminated soils [1,2,3]. However, *in situ* bioremediation trials show that this approach is not as successful under natural environmental conditions as would be expected from *in vitro* experiments [4,5]. One of the major reasons for this is the limited bioavailability of the pollutant, which in turn is a function of its hydrophobicity, solubility and persistence in the environmental matrix [4,5]. Increasingly, however, it has been recognized that microbial chemotaxis towards the pollutant can also be a major determinant [6,7,8,9]. Chloro-nitroaromatic compounds (CNACs) are a new class of toxic xenobiotic compounds that have been extensively used over the last few decades in the synthesis of pesticides, herbicides, dyes etc. Because of their stability, toxicity, mutagenicity and potential carcinogenicity, many CNACs, including chloro-nitrophenols (CNPs), chloro-nitrobenzenes (CNs) and chloro-nitrobenzoates (CNBs), have been listed as priority pollutants by organizations such as the United States Environment Protection Agency [10,11,12,13]. Microbial degradation could in theory be used to restore sites contaminated with CNACs but these compounds have proven to be extremely stable and recalcitrant to metabolic degradation [14] and there are very few reports of pure microbial isolates which are capable of degrading them [15,16,17,18].

2. Materials and Methods

Chemicals: All of the chemicals used were analytical grade (99% pure) or very pure (97% pure). All of the water used was ultrapure double-distilled water. 1C4NB, 4-chlorophenol, chlorobenzene, 4-nitrocatechol, and 4-chloroaniline, NAD1, NADH, and NADPH, 4-nitrophenol, 1, 2, 4-trihydroxybenzene and 4-chlorocatechol were obtained from Sigma Chemical Co. (St. Louis, Mo.); nitrobenzene and hydroquinone were obtained from E. Merck (Darmstadt, Germany); The trimethylsulfonium hydroxide (TMSH) reagent was obtained from Macherey-Nagel (Du¨ren, Germany). 2-Amino-5-chlorophenol and 5-chloropicolinic acid were prepared from 1C4NB by using 1C4NB-grown resting cells of strain XII as described below.

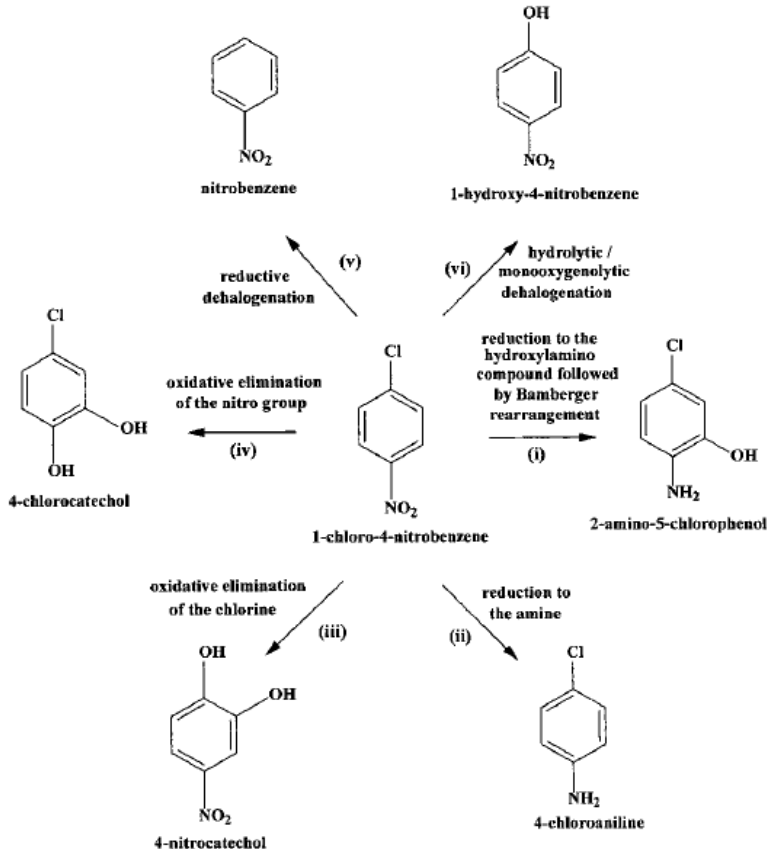

Figure 1. Putative initial reactions with 1C4NB based on studies of nitroben-zene degradation bia catechol (40) or 2-aminophenol (30),nitrobenzene trans-formation into aniline (34),reducative dehalogenation (13),and dehalogenation due to hydrolytic (25) or dioxygenolytic (32) activity

Bacterial strain and culture conditions. Strain XII was grown aerobically in a mineral salts medium (19) that was supplemented with 1C4NB as the carbon source and was incubated at 30°C on a rotary shaker at 150 rpm in baffled Erlenmeyer flasks. Due to its relatively low water solubility (20 mg/liter), 1C4NB was incubated with the mineral salts medium at 30°C and 150 rpm for 24 h, and the medium was filtered before inoculation to remove the undissolved crystals. Mineral salts medium containing dissolved 1C4NB (1.5 mM) was used to determine the degradation kinetics and mass balances. Scaled-up cultures were grown with an amount of 1C4NB that, if completely dissolved, corresponded to a concentration of 5 mM. The mineral salts medium was prepared as described previously [19]. For growth experiments performed with 1C4NB as the sole nitrogen source, Ca $(NO_3)_2$ was replaced by $CaCl_2$, Fe-ammonium citrate was replaced by $FeCl_2$ and (NH4) SO_4 was replaced by Na2SO4. Cultures were inoculated (10%, vol/vol) with precultures in the late exponential phase.

Preparation of cell extracts and resting cells. Cells of strain XII were harvested at the end of the exponential growth phase (optical density at 546 nm [OD546], 0.3). Cells were pelleted by centrifugation at 27,500 3 *g* for 10 min at 4°C, washed twice with 50 mM sodium phosphate buffer (pH 7.2), and resuspended in a small volume of the same buffer. The suspended cells were disrupted by four passages through a French pressure cell operated at 18,000 lb/in2. Intact cells and insoluble debris were removed by centrifugation at 40,000 *g* for 45 min at 4°C. The supernatant fluid was designated the cell extract and was used for enzyme assays. Experiments with resting cells were carried out with washed cells as described above; these cells were resuspended in 10 ml of 50 mM sodium phosphate buffer (pH 7.2) to a final OD_{546} of 5. Resting cells were incubated with 1.5 mM dissolved 1C4NB in a water bath shaker at 30°C.

Extraction, purification, and identification of metabolites. The metabolites 2-amino-5-chlorophenol and 5-chloropicolinic acid were purified and characterized as follows. 2-Amino-5-chlorophenol was prepared biologically from 1C4NB under anaerobic conditions in a glove box filled with oxygen-free nitrogen. The reaction mixture initially contained 50 mM sodium phosphate buffer (pH 7.2), 1.5 mM 1C4NB completely dissolved in the buffer, and 1C4NB-grown resting cells of strain XII. The reaction was carried out at 30°C with continuous stirring, and the progress of the reaction was monitored by monitoring the formation of 2-amino-5-chlorophenol by high-performance liquid chromatography (HPLC). Anaerobic conditions were necessary not only during the biotransformation but also during the workup. After 6 h of incubation and complete substrate turnover, the resting cells were pelleted by centrifugation, and the pH of the supernatant was adjusted to 12.0 by adding 5 N NaOH. Extraction was performed twice with ethyl acetate. The organic phase containing 2-amino-5-chlorophenol was concentrated by evaporation of the organic solvent under a vacuum after it was dried over Na_2SO_4, and it was analyzed without further purification by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Cell suspensions containing 2-amino-5-chlorophenol were aerated for 5 min for preparation of 5-chloropicolinic acid. After complete conversion, as determined by HPLC, the cells were pelleted by centrifugation. The pH of the yellow

supernatant was adjusted to 2.0 with HCl. The water phase lost its yellow color and was extracted twice with ethyl acetate. The organic phases were pooled, 5-chloropicolinic acid was concentrated by evaporation of the organic solvent under a vacuum, and the preparation was analyzed without further purification by NMR and GC-MS.

Enzyme assays. 2-Amino-5-chlorophenol 1, 6-dioxygenase activity was determined as previously described for the 2-aminophenol 1, 6-dioxygenase of *Pseudomonas pseudoalcaligenes [20]* by measuring the formation of the ring cleavage product of 2-amino-5-chlorophenol at 395 nm. The molar extinction coefficient (E395 5 21 mM21 cm21) was estimated by assuming that all of the 2-amino-5-chlorophenol was converted to the ring cleavage product under excess enzyme conditions as described previously [20]. 4-Nitrocatechol monooxygenase activities were measured as previously described [21]. Nitrobenzene reductase activity was measured as described previously [22] by monitoring the decrease in absorption at 340 nm due to conversion of NADPH to NADP. Catechol 1, 2- dioxygenase (EC 1.13.11.1), catechol 2, 3-dioxygenase (EC 1.13.11.2), muconate cycloisomerase (EC 5.5.1.1), and maleylacetate reductase (EC 1.3.1.32) activities were measured as previously described [23]. 1,2,4-Trihydroxybenzene 1,2-dioxygenase activity was determined qualitatively by monitoring spectral changes between 220 and 400 nm [24]. The reaction mixture contained 1, 2, 4-trihydroxybenzene (200 mM) and cell extract. Controls containing 50 mM phosphate buffer (pH 7.2) instead of cell extract were used to correct for autoxidation of 1, 2, 4-trihydroxybenzene. The oxygen uptake rates of resting cell suspensions washed twice with 50 mM sodium phosphate buffer (pH 7.2) were determined polar graphically by using a type LTD CB1D electrode. The rates were corrected for endogenous respiration. One unit of specific activity was defined as 1 mmol of substrate converted per min per gram of protein or 1 mmol of product formed per min per gram of protein at 25°C. The protein contents of cell extracts were determined by using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, Calif.), which is based on the colorimetric dye-binding procedure of Bradford [25]. The total protein content of XII cells was determined after lysis in the presence of 0.15 M NaOH at 100°C for 10 min and centrifugation at 27,500 *g* for 10 min to remove the cell debris; the protein assay described above was used.

Analytical methods. The chloride ion concentration was measured with a chloride sensor integrated into a flow-injection system as described previously [26]. The nitrite concentration was determined colorimetrically as described previously [27]. The ammonium ion concentration was measured colorimetrically by using a Spectroquant kit (E. Merck). The total organic carbon (TOC) contents of aqueous samples were determined by using a TOC analyzer (Shimadzu Corporation, Kyoto, Japan). Samples were centrifugated at 27,500 *g* for 10 min to remove the biomass and then acidified to pH 2.0 with a 2 N hydrochloric acid solution and degassed for 5 min with sparge nitrogen gas at a flow rate of 150 ml/min to remove the inorganic carbon dioxide. The TOC content was measured by the nonpurgeable organic carbon method. The inorganic carbon was first removed by catalytic conversion of all inorganic carbon to carbon dioxides, followed by nitrogen sparging. Calibration curves were obtained by using potassium hydrogen phthalate as the organic carbon standard. The HPLC analysis was carried out as follows. Water-soluble substrates and metabolites were analyzed with a Shimadzu HPLC system equipped with a type SC reversed-phase column (125 by 4.6 mm) filled with Lichrospher 100 RP8 5.0 mm (Bischoff, Leonberg, Germany). An aqueous solvent system containing 36 to 63% (vol/vol) methanol and 0.1% (vol/vol) *ortho*-phosphoric acid in Milli Q water was used as the mobile phase (flow rate, 1 ml/min). The column effluent was monitored simultaneously at 210 and 270 nm. The GC-MS analysis was carried out as follows. A model GC-17A gas chromatograph (Shimadzu) equipped with a type XTI-5 column obtained from Resteck (Bellefonte, Pa.) was used. A model QP-5000 quadrupole mass spectrometer was operated in the electron impact mode at 70 eV with an iron source temperature of 320°C. Helium was used as the carrier gas at a flow rate of 1.0 ml/min. The oven temperature was maintained at 60°C for 2 min, and then it was increased to 150°C at a rate of 20°C/min and finally to 320°C at a rate of 6°C/min. Samples (1.0 ml) were injected into the gas chromatography operating in the splitless mode with an injector temperature of 270°C. Methylation of metabolites was carried out with the TMSH reagent. One hundred microliters of the TMSH reagent was added to 50 ml of sample diluted in methanol, which was then boiled for 10 min (28).

Spectroscopic methods. High-resolution one-dimensional NMR spectra (1H, 13C) and two-dimensional NMR spectra (1H-detected long-range 13C-1H correlations) were recorded with a Bruker model DPX 300 NMR spectrometer locked onto the major deuterium resonance of the solvent, CD3OD. Chemical shifts (in parts per million) were determined relative to tetramethylsilane, and coupling constants (in hertz) were also determined.

3. Results

Isolation, characterization, and growth of 1C4NB-degrading strain XII. Strain XII was isolated from the activated sludge of common effluent tratemtn plant, india, by using a previously described enrichment technique [29] and 1C4NB as the sole carbon source. The taxonomy of this strain is currently under investigation. Results based on 16S rRNA sequencing showed that strain XII belongs to *Pseudomonas* family. Strain XII utilized 1C4NB as a sole carbon, nitrogen, and energy source. A growth curve obtained with 1C4NB as the only substrate is shown in Figure 2. The initial concentration of the dissolved carbon source in the culture fluid was 1.3 mM. Doubling times of 4 to 6 h were observed during the exponential growth phase. The amount of chloride released (1.3 mM) indicates that stoichiometric elimination occurred. Accumulation of ammonium or nitrite was not observed, as determined by the colorimetric assay, nor was metabolite accumulation, as determined by HPLC analysis. The initial amount of TOC was reduced by 75%. Growth of strain XII with substrate analogues, such as nitrobenzene and chlorobenzene, and with several hypothetical pathway intermediates, such as 2-amino-5-chlorophenol (which was biologically prepared in this

work), 4-chloroaniline, 4-nitrophenol, 4-nitrocatechol, 4-chlorophenol, and 4-chlorocatechol, was tested in liquid cultures by using each compound at a concentration of 1 mM, as well as on diffusion gradient agar plates, which should have prevented toxic effects on bacterial cells. Only two of these compounds, nitrobenzene and 2-amino-5-chlorophenol, were utilized as carbon sources by XII.

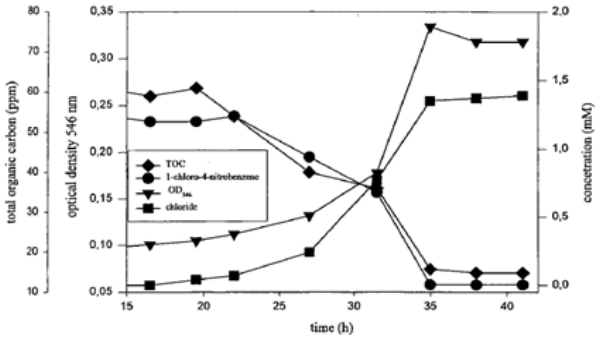

Figure 2. Growth of bacterial strain XII with 1C4NB as the sole carbon source.The initial concentration of the dissolved carbon source in the culture fluid was 1.2 mM. The culture was inoculated (10%, vol/vol) with a preculture grown with 1C4NB and harvested during the exponential phase. Substrate depletion, formation of chloride, and TOC values were determined as described in Materials and Mdthods.Growth was monitored by monitoring the increase inOD_{546}

Aerobic transformation of 1C4NB and of hypothetical pathway intermediates. In order to elucidate the pathway of 1C4NB degradation in strain XII, oxygen uptake experiments were performed with growth substrates and several hypothetical intermediates (Table 1). 1C4NB-grown resting cells had an oxygen uptake rate of 86 U/g of protein with 1C4NB, and the oxygen uptake rate with 4-chlorocatechol was approximately fourfold lower (23 U/g of protein). Negligible oxygen consumption occurred with 4-chlorophenol, 4-nitrocatechol, 4-nitrophenol, hydroquinone, and 4-chloroaniline. In contrast, the specific transformation rate (23 U/g of protein) for 1C4NB (1.3 mM) obtained with 1C4NB-grown resting cells (OD546, 6.0), as determined by HPLC, was lower than the specific oxygen uptake rate. Again, there was no indication that significant amounts of pathway intermediates accumulated, as determined by HPLC. Stoichiometric amounts of both chloride (1.4 mM) and ammonium (1.4 mM) accumulated, as determined by colorimetric assays (Figure 3). Similar results were obtained with acetate-grown cells, which transformed 1C4NB at a rate of 20 U/g of protein. 4-Chlorocatechol was transformed by resting cells at a rate of 18 U/g of protein, which was similar to the measured oxygen uptake rate. Transformation was accompanied by transient yellow coloration of the medium. HPLC analysis of the supernatant revealed that there was transient formation of a metabolite, the in situ UV spectrum (after HPLC) of which exhibited an absorption maximum at 332 nm. Formation of small amounts of a new product from 4-chlorocatechol by both 1C4NB-grown resting cells and acetate-grown resting cells was detected by GC-MS of methylated ethyl acetate extracts; this new product was later identified as 5-chloropicolinic acid. When 4-nitrophenol and hydroquinone were used, about 20% of the substrates were removed from the resting cell suspensions in 6 h, but no products were detected by HPLC. No significant conversion was observed with 4-chloroaniline or 4-nitrocatechol. While there was no significant decrease in the initial concentration of 4-nitrocatechol (200 mM), a quick change in the color of the reaction mixture from yellow to orange took place. No reaction was observed with controls that did not contain cells or controls that contained cells that had been heat inactivated (10 min, 60°C). The activities of various possible pathway enzymes in cell extracts are shown in Table 2. Low nitrobenzene reductase activities, 2 and 22 U/g of protein with nitrobenzene and 1C4NB as the substrates, respectively, were observed. In contrast to the observed transformation by whole cells, the cell extracts exhibited neither catechol 1, 2-dioxygenase activity nor catechol 2, 3-dioxygenase activity when 4-chlorocatechol or catechol was the substrate. No muconate cycloisomerase and chloromuconate cycloisomerase activities were observed when muconate and 2-chloromuconate were the substrates, respectively. No 4-nitrocatechol monooxygenase activity was observed. The spectral changes observed with cell extracts when 1, 2, 4-trihydroxybenzene was the substrate indicated that a 1, 2, 4-trihydroxybenzene 1, 2-dioxygenase activity was present. However, no activity of the potential subsequent pathway enzyme maleylacetate reductase was observed.

Table 1. Relative oxygen uptake rates and conversion rates for possible pathway intermediates in 1C4NB-grown resting cells

Substrate	Reaction[a]	Specific rate of oxygen consumption (U/g of protein) after growth with 1C4NB[b]	Initial conbersion rate (U/g of protein)[c]
1C4NB		86	23
2-Amino-5-chlorophenol	i	450	820
2- Aminophenol		160	290
4-Chloroaniline	ii	<1	0
4-Nitrocatechol	iii	160	0
4-Nitrophenol	iii	<1	1.3
Hydroquinone	iii	1	0.7
4-Chlorocatechol	iv	23	18
4-Chlorophenol	iv	1	ND[d]
Nitrobenzene	v	43	ND
Chlorobenzene		7	ND

[a]Ctabolic pathway in which the compound is apossible intermediate. See Figure 1.
[b]The oxygen uptake rates were corrected for endogenous respiration.Most substrates were added at an initial concentration of 100 Um; the exceptions were centration of 1.0 mM.
[c]In most cases depletion was measured by HPLC for 6h; for 2-aminophenol and 2-amino-5-chlorophenol depletion was measured for 30 min. Experiments wew carried out under aerobic conditions. Most substrates were added at an 1.3 mM. With the exception of 4-chlorocatechol transformation, no products were detected by HPLC.
[d]ND, not determined.

Table 2. Specific activities of catabolic enzyme activities in cell extracts of strain XII

Enzyme[a]	Reaction (s)[b]	Substrate	Sp act after growth with 1C4NB (U/g of protein)
Nitrobenzene reductase	i	Nitrobenzene	2
		1C4NB	22
2-Amino-5-chlorophenol 1,6-dixoxygenase	i	2-Amino-5-chlorophenol	3,570
2-Aminophenol 1,6-dixoxygenase		2-Aminophenol	3,020
4-Nitrocatechol monooxygenase	iii	4-Nitrocatechol	<0.01
Maleylacetate reductase	iii, iv	Maleylacetate	<0.01
Catechol 1,2-dioxygenase	iv	Catechol	<0.01
		4-Chlorocatechol	<0.01
Catechol 2,3-dioxygenase	iv	Catechol	<0.01
		4-Chlorocatechol	<0.01
Chloromuconate cycloisomerase	iv	2- Chloromuconate	<0.01
Muconate cycloisomerase	iv	Muconate	<0.01

[a]Cell extract was added tto give final protein concentrations of 0.1 to 0.15 mg. Enzyme assays were performed as described in Materials and Methods.

[b]See Figure 1.

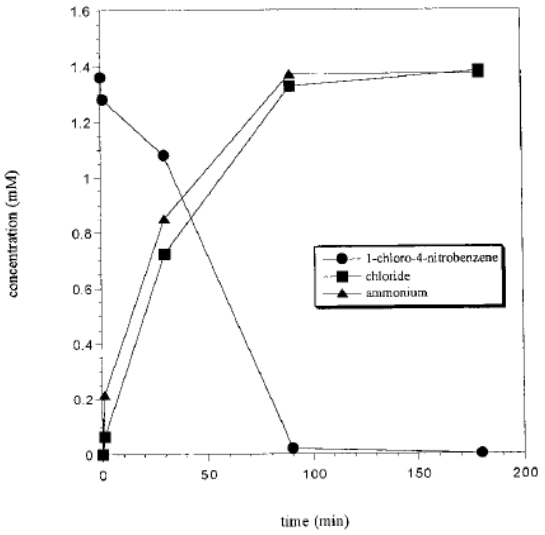

Figure 3. Time course for conversion of 1C4NB by resting cells of strain XII grown with 1C4NB under aerobic conditions. Cell suspensions at OD_{546} of 6.0 was added to a final concentration of 1.36 mM. Substrate depletion was determined by HPLC

Anaerobic transformation of 1C4NB by 1C4NB-grown resting cells. Previous results indicated that the nitro substituent was reduced, that ammonium rather than nitrite was eliminated, and that a nitrobenzene reductase was present; therefore, transformation of 1C4NB in the absence of oxygen should have led to the accumulation of pathway intermediates. Under anaerobic conditions, XII cells grown on 1C4NB converted 1C4NB into a single product (Figure 4), later identified as 2-amino-5-chlorophenol. The elimination of chloride or ammonium under these conditions was negligible. Acetate-grown resting cells of XII converted 1C4NB under anaerobic conditions at the same conversion rate. The metabolite mentioned above had a retention volume of 3.5 ml when an HPLC solvent system containing 36% (vol/vol) methanol was used and had an in situ UV absorption spectrum with maxima at 202, 217, and 277 nm at pH 2.0. The mass spectrum of its methyl ether had a molecular ion at *m*/*z* 5 158 (M1), indicative of C7H8C1NO, and major fragments due to the loss of 2CH3 (*m*/*z* 5 142) and 2CH3, 2CO (*m*/*z* 5 114). The 1H and 13C NMR data (Table 3) were unambiguously assigned on the basis of the two-dimensional 1H-detected longrange 13C-1H correlation. The 13C chemical shift data were compatible only with the substitution pattern of 2-amino-5-chlorophenol, as shown by calculations of the shifts when the known shifts of 2-aminophenol [30] and the chlorosubstituent chemical shifts (SCS) were used, and they were incompatible with the substitution pattern of 2-chloro-5-aminophenol (with known shifts of 4-chloroaniline and SCS of an aromatic hydroxyl group [31]. Assuming that the new product was 2-amino-5-chlorophenol, we calculated that transformation of 1C4NB was stoichiometric and occurred at a rate of 4.5 U/g of protein.

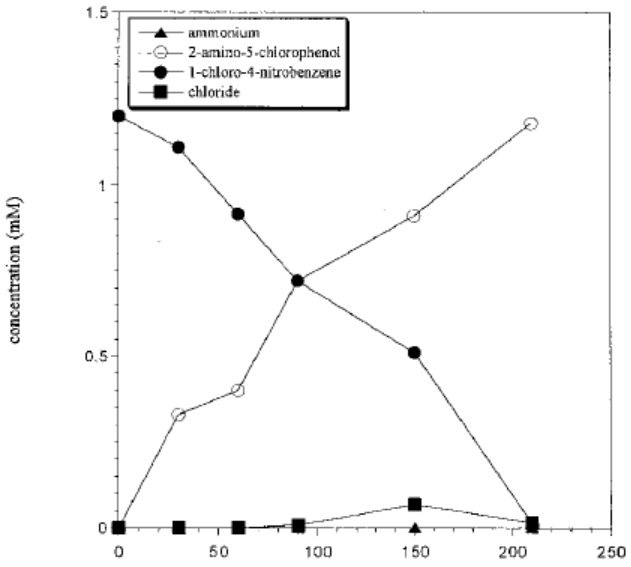

Figure 4. Time course for conversion of 1C4NB by resting cells of strain XLL grown with 1C4NB under anserobic conditions. Cell suspensions at an OD_{546} or 5.0 were resuspended in 50 Mm sodium phosphate buffer (pH 7.2). Substrate was added to a final concentration of 1.2 mM. Substrate depletion and product formation were determined by HPLC

Aerobic transformation of 2-amino-5-chlorophenol. 2-Amino-5-chlorophenol (initial concentration, 0.575 mM) was rapidly converted into a yellow product by 1C4NB-grown resting cells at a conversion rate of 820 U/g of protein. An HPLC analysis revealed that a single product was formed, and this product had a retention volume of 4.2 ml and an in situ UV spectrum with absorption maxima at 201, 234, and 274 nm. This compound was extracted and derivatized as described above. The mass spectrum of the methyl ester had a molecular ion at *m*/*z* 5 171 (M1), indicative of C7H6C1NO2. Major fragments appeared at *m*/*z* 5 141 (M1 2OCH2) and at *m*/*z* 5 113 (M1

2CO2CH2). Loss of HCl from the mass at 112 yielded a mass of 76, which is consistent with the presence of a pyridine nucleus. These results are similar to the previously published mass spectrum of the methyl ester of 2-chloropicolinic acid [32]. Again, unambiguous NMR assignments (Table 3) were provided by the two-dimensional 1H-detected long-range 13C-1H correlation, and both 13C shift values (with picolinic acid [33] and chloro SCS) and the magnitude of the one-bond 13C-1H coupling constants were compatible only with the proposed structure. The transformation of 2-amino-5-chlorophenol into 5-chloropicolinic acid was not stoichiometric; only 50% of the substrate was recovered as 5-chloropicolinic acid (0.267 mM). Despite the fact that no other products were detected by HPLC, the accumulation of significant amounts of ammonium (0.124 mM) but negligible amounts of chloride (0.043 mM) indicated that additional products were formed. 1C4NB-grown resting cells of XII had an oxygen uptake rate of 450 U/g of protein when 2-amino-5-chlorophenol was the substrate, which was fivefold higher than the oxygen uptake rate observed with 1C4NB. Resting cells had very low oxygen uptake rate (8 U/g of protein) when 5-chloropicolinic acid was the substrate.

Table 3. ^{1}H and ^{13}C NMR data for 2-amino-5-chlorophenol and 5-chloropicolinic acid in CD_3OD

2-Amino-5-chlorophenol			5-chloropicolinic acid			
^{1}H chemical shifts (ppm)	^{1}H-^{1}H coupling constants (Hz)	^{13}C chemical shifts (ppm)	^{1}H chemical shifts (ppm)	^{1}H-^{1}H coupling constants (Hz)	^{13}C chemical shifts (ppm)	One-bond ^{13}C-^{1}H coupling constats (Hz)[a]
H-3 6.70	(3-4) 8.3	C-1 147.2	H-3 8.18	(3-4) 8.4	C-2 147.6	
H-4 6.64	(4-6) 2.3	C-2 135.5	H-4 8.08	(3-6) 0.7	C-3 127.3	168.0
H-6 6.72		C-3 117.6	H-6 8.70	(4-6) 2.4	C-4 138.7	170.5
		C-4 120.5			C-5 137.1	
		C-5 123.8			C-6 149.4	186.3
		C-6 115.4			C-7 166.6	

[a]The values of the one-bond ^{13}C-^{1}H coupling constats were estimated from the residual cross peaks in the ^{1}H-detected long range ^{13}C-^{1}H correlation.

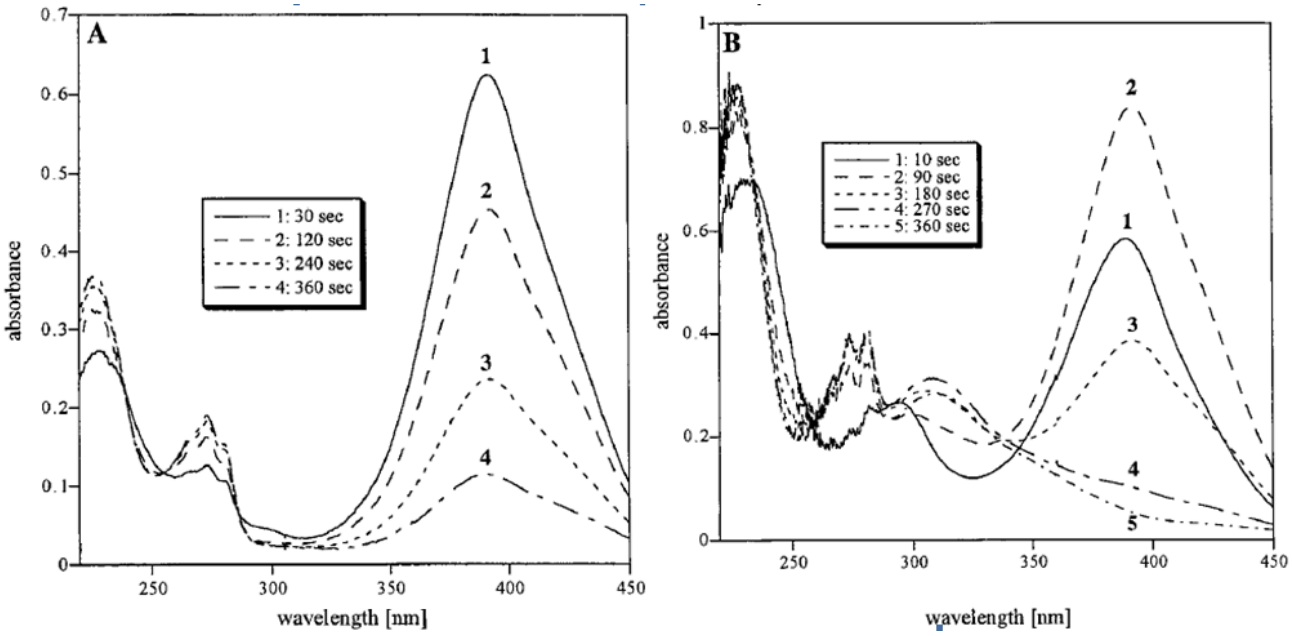

Figure 5. Spectral changes due to 2-amino-5-chlorophenol 1,6-dioxygenase activity in extracts of cells pregrown with 1C4NB. (A) Reaction mixture containing 2-amino-5-chlorophenol (100 uM) and XII cell extract (0.1 mg of protein) in 1 mi of 50 mM sodium phosphate buffer (pH 7.2). (B) Reaction mixture also containing NAD (100 uM). Overlay spectra were recorded for 6 min from 220 to 450 nm as indicated

2-Amino-5-chlorophenol 1, 6-dioxygenase activity. Cell extracts catalyzed transformation of both 2-aminophenol and 2- amino-5-chlorophenol, and yellow intermediates with absorption maxima at 380 and 395 nm, respectively, appeared and disappeared rapidly. Figure 5A shows the changes in the UV visible light spectra during enzyme reactions when 2-amino-5-chlorophenol was the substrate. When the initial maximum absorption at 395 nm decreased, absorption at 226 and 272 nm

increased. The final absorption spectrum was identical to that of 5-chloropicolinic acid. Cell extracts of XII catalyzed transformation of 2-amino-5-chlorophenol and 2-aminophenol with specific activities of 3,570 and 3,020 U/g of protein, respectively. Figure 5B shows the changes in the UV-visible light spectra during transformation by cell extracts of 2-amino-5-chlorophenol in the presence of NAD, which differed from the results of spectrophotometric assays in which NAD was not present by the presence of a new absorption maximum at 306 nm.

4. Discussion

The accumulation of ammonia but not of nitrite in conversion experiments performed with resting cells grown with 1C4NB and the observed 1C4NB reductase activity in XII cell extracts suggested that the initial attack on the nitro group was reductive. The complete conversion of 1C4NB to 2-amino-5-chlorophenol in the absence of oxygen indicated that the nitro group was partially reduced. In the proposed pathway, the nitro group is partially reduced to a hydroxylamino group; this is followed by an enzyme-catalyzed Bamberger rearrangement to form 2-aminophenol (20), whereas a

nonenzymatic Bamberger rearrangement results in the formation of the corresponding 4-aminophenol [34]. Formation of 2-aminophenol as the only intermediate has also been reported for nitrobenzene degradation by *Pseudomonas pseudoalcaligenes* JS45 [20] and for 4-nitrotoluene degradation by a *Mycobacterium* strain [35]. Similarly, 3-nitrophenol was transformed by *Ralstonia eutropha* JMP 134 into aminohydroquinone but not into 4-aminocatechol [36]. In contrast to the high regioselectivity of the rearrangement observed during transformation of the growth-supporting substrate 3-nitrophenol, nitrobenzene, which does not serve as a growth substrate, was transformed into both 2-aminophenol and 4-aminophenol by this strain [36]. It has been assumed that a similar reaction occurs during transformation of 2, 4, 6-trinitrotoluene by *Clostridium acetobutylicum* [37]. 2-Amino-5-chlorophenol, but not 4-amino-5-chlorophenol, was formed as an intermediate during degradation of 1C4NB by XII. Evidently, in microorganisms able to mineralize nitroaromatic compounds via a pathway involving Bamberger rearrangement, this rearrangement leads exclusively to the formation of 2-aminophenols, which are subject to *meta* cleavage. The transformation of 2-aminophenol by XII cell extracts was similar to the transformation of 2-aminophenol by *P. pseudoalcaligenes* JS45 1, 6-dioxygenase [20]. The ring cleavage product cyclized spontaneously to form picolinic acid. Similarly, the ring cleavage product of 2-amino-5-chlorophenol with a UV maximum at 395 nm (probably 2-amino-5-chloromuconic semialdehyde) intramolecularly condensed to give 5- chloropicolinic acid. As this compound was not a growth substrate and was not transformed further by whole cells or cell extracts, a degradative pathway involving this compound as an intermediate is highly unlikely. Such abiotic chemical rearrangements may take place when an unstable intermediate is formed and the activity of the subsequent pathway enzyme is insufficient under the conditions used. When 2-amino-5-chlorophenol was the substrate, high transformation rates were observed. A pathway bottleneck presumably prevented the complete conversion of the semialdehyde formed, which led to the accumulation of 5-chloropicolinic acid. In contrast, when 1C4NB was the substrate, the rate-limiting initial reduction (low 1C4NB reductase activity) suggests that the rate of formation of the semialdehyde was low, so that additional pathway enzymes could quantitatively channel the intermediate into the productive route. In accordance with this hypothesis, only traces of 5-chloropicolinic acid (50 mM) accumulated in both growing and resting XII cultures when 1C4NB was used as the substrate. Formation of picolinic acid as a dead-end product has been reported in many studies, and this compound has been shown to arise from abiotic intramolecular condensation of enzymatically formed 2-aminomuconic semialdehyde derivatives [35,38,39]. The chemical synthesis of various picolinic acid derivatives from ammonia and 2-hydroxymuconic semialdehyde derivatives which had been biologically prepared from various catechols by using catechol 2, 3-dioxygenase [40]. Similarly, The abiotic formation of halopicolinic acids from substituted 2-halohydroxymuconic semialdehydes formed during degradation of chloro- and bromobiphenyls by *Sphingomonas paucimobilis* BPSI-3 [32]. Riegert et al. used this abiotic transformation to determine the structure of the ring fission product of 3-chlorocatechol obtained during 2, 3-dihydroxybiphenyl 1, 2-dioxygenase activity of *Sphingomonas* sp. strain BN6 [41]. The nitrogens present in picolinic acid formed from 2-aminophenol by *P. pseudoalcaligenes* JS45 [20] and in 5-chloropicolinic acid formed from 2-amino-5-chlorophenol by XII, however, clearly originated from the substrates. The high activities observed with both 2-aminophenol and 2-amino-5-chlorophenol in XII cell extracts indicated that a 2-amino-5-chlorophenol 1, 6-dioxygenase with high substrate specificity was present. Neither catechol nor 4-chlorocatechol was transformed by cell extracts. Similar substrate specificity was observed for the 6-amino-*m*-cresol 1, 6-dioxygenase of a *Mycobacterium* strain which was shown to be active against 2-aminophenol and the pathway intermediate 6-amino-*m*cresol but not against catechol or 4-methylcatechol [35]. In contrast, the 2-aminophenol 1, 6-dioxygenase of *P. pseudoalcaligenes* JS45 exhibited significant activity with catechol [39]. Additional substituents in both the 2-aminophenol structure and the catechol structure severely diminished or abolished activity. 2-Aminophenol 1, 6-dioxygenase of *Pseudomonas* sp. strain AP-3 also catalyzed the catechol reaction (38). The amino acid sequence of *b*-subunit AmnB of purified 2-aminophenol 1, 6-dioxygenase of *Pseudomonas* sp. strain AP-3 exhibited similarities to the amino acid sequences of extradiol dioxygenases [42]. Lendenmann and Spain suggested that the purified 2-aminophenol 1, 6-dioxygenase of *P. pseudoalcaligenes* JS45 is also related to catechol 2, 3-dioxygenases. How much differences in substrate specificity are reflected by evolutionary relationships still must be elucidated. The catabolic pathway responsible for transformation of 1C4NB (Figure 6) seems to be constitutively expressed in XII, as similar conversion rates for 1C4NB and 2-amino-5-chlorophenol were obtained with 1C4NB-grown cells and acetate-grown cells (data not shown). This contrasts with the situation in recently described 3-nitrophenol- and 4-nitrotoluene-degrading organisms [36], whose cells did not transform the respective substrates when they were grown under noninducing conditions. As described above, extracts of 1C4NB-grown cells of XII, like extracts of nitrobenzene-grown cells of *P. pseudoalcaligenes* JS45, transformed 2-aminophenol into picolinic acid [20]. In both cases addition of NAD to reaction mixtures containing 2-aminophenol and cell extracts reduced the formation of picolinic acid and resulted in transient accumulation of NADH and formation of 2-aminomuconate [43]. In *P. pseudoalcaligenes* JS45, 2-aminomuconate was further transformed by 2-aminomuconate deaminase into 2-oxohex-3-ene-1, 6-dioate, which, in turn, can be assumed to be degraded to Krebs cycle intermediates by enzymes of the classical *meta*-cleavage pathway [43]. It has been postulated that there is an alternative pathway for 2-aminophenol degradation in *Pseudomonas* sp. strain AP-3 [44]. Takenaka et al. postulated that there is an initial decarboxylation of the intermediate 2-aminomuconic acid, based on identification of 2-aminopenta-2, 4-dienoic acid as an intermediate, which is subject to deamination, resulting in 2-oxopent-4-enoic acid. Whereas spectrophotometric monitoring of the progress of 2-aminophenol transformation by XII cell extracts in the presence of NAD gave no indication that pathway

intermediates were formed, a product exhibiting an absorption maximum at 306 nm accumulated during 2-amino-5-chlorophenol transformation. This product interfered with spectrophotometric monitoring of the accumulating NADH. If it is assumed that there is a pathway in XII for the degradation of 2-amino-5-chlorophenol similar to the pathway characterized by He and Spain and Takenaka et al. for 2-aminophenol degradation, it can be assumed that 2-amino-5-chloromuconate and 5-chloro-2-oxohex-3-ene-1,6-dioate or 2-aminopenta-2,4-dienoate are intermediates. 2-Aminomuconate has been reported to exhibit an absorption maximum at 326 nm [43]. Whether the new absorption maximum at 306 nm can be attributed to transient accumulation of 2-amino-5-chloromuconate or transient accumulation of 5-chloro-2-hydroxymuconate will be the subject of further investigation. Although the growth substrate 1C4NB is transformed by XII at a low rate, the pathway intermediate 2-amino-5-chlorophenol is transformed very rapidly, so that subsequently, active pathway enzymes cannot adequately turn over the intermediates. In addition to the accumulation of 5-chloropicolinic acid, the increase in absorbance at 306 nm mentioned above indicates that there is a second pathway bottleneck. Moreover, the observed elimination of larger amounts of ammonium than of chloride during resting cell-mediated transformation of 2-amino-5-chlorophenol could indicate that ammonium elimination precedes dechlorination. The next steps of the pathway, including those responsible for elimination of ammonium and chloride in XII, are currently under investigation.

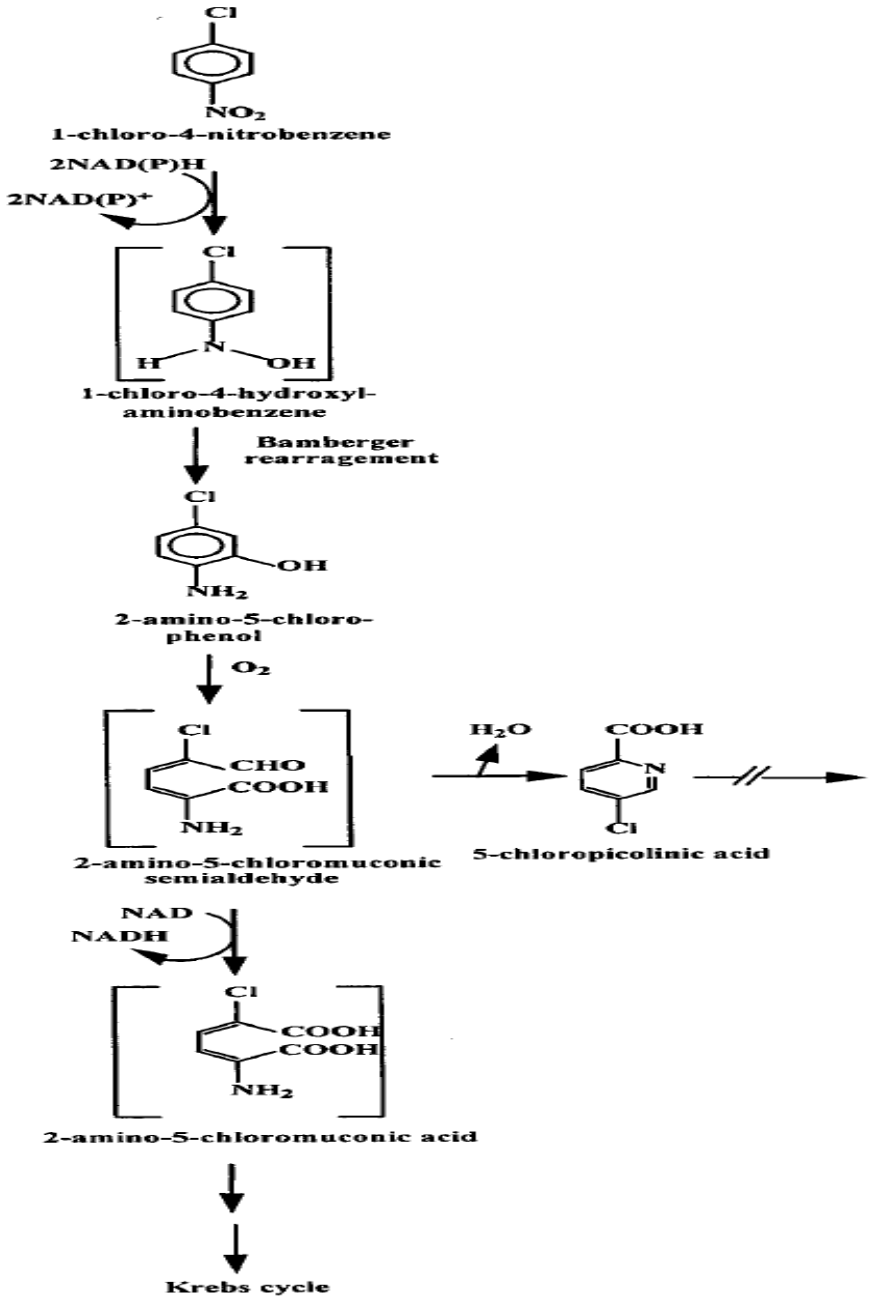

Figure 6. Proposed pathway for catabolism of 1C4NB by bacterial strain XII. The ring cleavage product 2-amino-5-chloromuconic semialdehyde can be subject to dehydrogenation (probably producing 2-amino-5-chloromuconic acid) or intramolecular condensation to form the dead-end product 5-chloromuconic acid as previously reported for 2-aminophenol transformation by *P. pseudoalcaligenes* JS45 (20) or by *Pseudomonas* sp. strain AP-3 (48)

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