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Microbial metabolism of chloroanilines: enhanced evolution by natural genetic exchange

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Abstract. 2-, 3-, and 4-chloroaniline degrading bacteria were obtained by natural genetic exchange between an aniline or toluidine degrading *Pseudomonas strain* and the chlorocatechol assimilating *Pseudomonas* sp. B13. Hybrid organisms were isolated through cocultivation of the parent strains in the chemostat as well as through conjugation on solid media in presence of chloroanilines as the selective substrates. Biochemical analysis of the gene products in the hybrid strains clearly showed that the genes coding for the aniline dioxygenase or the genes for the chlorocatechol assimilatory sequence had been transferred.

Key words: Chloroaniline utilization – Hybrid organisms – Chlorocatechol degrading enzymes – *Pseudomonas*

Chlorinated anilines are intermediates and building blocks of the large scale synthesis of herbicides, dye stuffs and pharmaceuticals. The environmental problems that may arise from a widespread use of these chemicals are twofold. Firstly, the chlorinated aromatic ring confers some xenobiotic character to pesticides such as chlorophenylcarbamates, chlorophenylureas, and acylchloroanilides, which are purposely introduced into the environment in a dispersed form. Thus the major part of these synthetic chemicals are present in the biosphere at relatively low concentrations. Insufficient knowledge is presently available to describe the fate of chloroaniline derivatives at low concentrations, although some investigations (Chisaka and Kearney 1970; Bartha 1971; Kaufman and Blake 1973; Deuel et al. 1977) indicate that fortuitous metabolic processes generate chlorinated anilines as microbial metabolites.

These chloroanilines are rather recalcitrant in soils and aquatic environments because coupling to humic substances (Bartha 1980) or oxidative dimerisation yielding chlorosubstituted biphenyls, azobenzenes or triazenes (see Corke et al. 1979) slows biodegradation of these xenobiotics. Consequently these experiments strongly suggest that the fate and environmental quality of these chemicals in the open environment is largely governed by fortuitous metabolic reactions which cannot be accelerated biotechnologically.

A second environmental problem arises from rather high concentrations of free chloroanilines as critical components of industrial effluents. These are generated by cometabolic transformations e.g. hydrolytic cleavage of acyl derivatives of chloroanilines or reduction of nitrochlorobenzenes. If conventional sewage treatment systems are exposed to high loads of these xenobiotics the effluents of the settling tank exhibit a brown black coloration and contain high concentrations of dissolved organic carbon. The bulk of this undecomposed organic matter is due to secondary hydroxylated and polymerized products and cannot be correlated to intact chloroanilines or defined metabolites. Investigations with soil populations and pure cultures indicate that ordinary aniline degrading bacteria cooxidize relatively high concentrations of chloroanilines to chlorocatechols (Reber et al. 1979; Schukat et al. 1983) and further to 2-hydroxy-5-chloromuconic semialdehyde (Surovtseva et al. 1980). In principle chloroanilines can be subject to microbial attack, however, accumulation of dead end metabolites such as chlorocatechols and their colored polymerisation products or misrouting into unproductive pathways (e.g. *meta*-cleavage pathways) presents total degradation of these xenobiotics. This together with the inherent toxicity of these chlorinated aromatics explains why chloroaniline utilizing bacteria are not readily isolated from existing industrial sewage treatment systems. Only recently a *Pseudomonas* strain was isolated from soil by chemostat enrichment which grew slowly on 4-chloroaniline as a sole source of carbon and nitrogen (Zeyer and Kearney 1982).

A number of adaptation procedures can be deviced in the laboratory for circumvention of critical and unproductive catabolic steps. Here we demonstrate the possibility of enhanced evolution of chloroaniline degrading bacteria through strain construction by natural genetic exchange. An aniline and toluidine utilizing bacterium, which cometabolizes chloroanilines via catechols, and *Pseudomonas* sp. B13 with its chlorocatechol degrading capability were used as parent strains carrying complementary catabolic routes. Hybrid organisms capable of growth with chloroanilines as sole source of carbon and energy were readily isolated from chemostat enrichments as well as by conjugation on solid medium containing the xenobiotic as the selective substrate.

Materials and methods

Culture conditions

For growth in liquid culture, the mineral medium as described by Dorn et al. (1974) contained the respective substrate at concentrations as indicated in the section "Results". Higher substrate concentrations inhibited exponential growth. Small quantities of cells were grown in 500 ml-

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Erlenmeyer flasks containing 50 ml of medium. The flasks were incubated at 28° C on a rotary shaker at 150 rpm. Large-scale growth of biomass was carried out in a 2 l fermentor (New Brunswick, Edison, NJ, USA), containing 1 l of medium. Air was introduced at a rate of 0.4 l/min and the cultures were stirred at 400 rpm at 30° C.

For continuous culture the cells were grown in an apparatus as described by Hartmann et al. (1979).

Solid media were prepared by addition of 2% Ionagar (Oxoid) no. 2 to solutions of the basal medium. Stock cultures were maintained on nutrient agar slopes, subcultured monthly, and stored at 20° C. Cells were harvested during exponential growth phase by centrifugation (10,000 rpm, 20 min at 10° C) suspended in 33 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0). Oxygen uptake experiments were carried out with freshly harvested cells that were centrifuged and washed once with 50 mM phosphate buffer (pH 7.4) and resuspended in the same buffer.

Preparation of cell-free extracts

Cell suspensions in 33 mM tris-hydrochloride buffer, pH 8.0, were disrupted by using a French press (Aminco, Silver Spring, MD, USA). Cell debris was removed by centrifugation at 30,000 × *g* for 20 min.

Enzyme assays

Catechol 2,3-dioxygenase (EC 1.13.11.2) was assayed by the method of Nozaki (1970). Catechol 1,2-dioxygenase (EC 1.13.11.1) activity was measured by the procedure of Dorn and Knackmuss (1978a, b). Extinction coefficients for the ring fission products were those reported by Dorn and Knackmuss (1978b).

Muconate cycloisomerase (EC 5.5.1.1), 4-carboxymethylenebut-2-en-4-olide hydrolase, 2-hydroxymuconic semialdehyde hydrolase and dehydrogenase were assayed by using previously described procedures (Ornston 1966; Schmidt and Knackmuss 1980; Williams and Murray 1974).

The activities were measured immediately after preparation of the extracts. Assays were carried out at saturating levels of substrate so that the reaction rates were proportional to protein content. One unit of enzyme activity was defined as the amount of enzyme required to produce or convert 1 µmol of product or substrate per min. Specific activities were expressed as units per gram of protein at 25° C. Protein content was determined by the method of Bradford (1976).

Rates of oxygen uptake were measured polarographically by use of a Clark oxygen electrode. Freshly harvested cells were washed and suspended in 50 mM phosphate buffer (pH 7.4) to an optical density of 1.0 at 546 nm. Cell suspensions were saturated with air in the cuvettes. After 5 min of constant endogenous oxygen uptake, the reaction was started by injecting 10 µmol of assay substrate. Uptake rates were determined with beginning time course curves and corrected for endogenous O_2 uptake. Activities are expressed in micromol of O_2 uptake per min per ml of cell suspension ($OD_{546\,nm}$ 1.0).

Cooxidation of methylanilines

Chloroaniline-grown cells were washed and resuspended in 50 mM phosphate buffer (pH 7.4) to an optical density of 10 at 546 nm. Cells were incubated with the respective methylanilines in 250 ml Erlenmeyer flasks at 30° C on a rotary shaker. Production of cooxidation products was followed by high pressure liquid chromatography. Compounds were identified with authentic samples by their retention times and in situ scanning of their ultraviolet spectra.

Analytical methods

Substituted anilines in the culture fluid were analyzed by reverse-phase high pressure liquid chromatography (chromatography model M-6000A, Waters, USA; Micro Pak CH-column, 250 mm by 2 mm, octadecylsilane chemically bonded to LiChrosorb 10 µ particles). The mobile phase was MeOH (1,170 ml), ammonium acetate (33.6 g) and water (ad 2,000 ml). A variable-wavelength spectrometric detector (model SF 770, Schoeffel, Westwood, NY, USA) and a computing integrator (Autolab System I, Spectra-Physics, Santa Clara, CA, USA) were used for quantification. Samples of culture fluids (5 to 20 µl) were injected after cells had been removed by centrifugation in 0.5 ml micro-test tubes for 3 min at 20,000 rpm (Mikro-Hämatokrit, Heraeus Christ, Osterode, FRG).

Chloride ion concentration were measured with an ion selective combination chloride electrode (model 96/17, Orion Research Inc., Cambridge, MA, USA), which was calibrated with NaCl (0.1 mM up to 50 mM) in mineral medium before each measurement.

Chemicals

3-Chlorobenzoic acid, aniline, 2-methyl-, 2-chloro-, 4-chloro-, and 3,4-dichloroaniline were obtained from Fluka AG, Buchs, Switzerland. Catechol was purchased from E. Merck AG, Darmstadt, FRG, whereas 3-methyl- and 4-methylcatechol were obtained from EGA Chemie, Steinheim, FRG. 3-Chloro- and 4-chlorocatechol were prepared as previously described (Schreiber et al. 1980). 3,5-Dichlorocatechol was synthesized by chlorination of 2-hydroxybenzaldehyde by the method of Biltz and Stepf (1904) and by subsequent Darkin reaction (Azouz et al. 1955). The catechols were purified by sublimation before use.

2-Hydroxy-6-oxohepta-2,4-dienoate and 2-hydroxy-5-methyl-6-oxohexa-2,4-dienoate were prepared enzymatically from 3-methyl- and 4-methylcatechol, respectively, by using purified catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2. *cis, cis*-Muconic acid was synthesized by the method of Wacek and Fiedler (1949). 2-Chloromuconic acid, 4-carboxymethylenebut-2-en-4-olide, 2-methyl- and 4-methyl-4-carboxymethylbut-2-en-4-olide were prepared as described elsewhere (Schmidt et al. 1980).

All other chemicals employed were of analytical grade and obtained from Merck AG, Darmstadt, FRG. Biochemicals were obtained from Boehringer AG, Mannheim, FRG.

Results

Isolation and growth of a Pseudomonas strain capable of growth with aniline and 2-methylaniline (2MA)

Preliminary experiments had shown that 2-methylaniline (o-toluidine) was readily degraded by microbial populations from soil whereas scant growth was observed with the isomeric 3- or 4-methylanilines. Therefore 2-methylaniline

(2MA) was used as a selective substrate in order to enrich strains that induce aniline dioxygenases with relaxed substrate specificities. A 2MA-utilizing population was obtained from a mixture of soil samples from the Göttingen area through batch culture enrichment with 2MA (2mM) as sole carbon source. Single isolates were obtained by streaking the cultures on mineral salts agar containing 5 mM 2MA. A pure culture, strain JL1, was derived from a well grown colony by repeated streaking on 2MA-agar plates. This organism exhibited the phenotypic characters of *Pseudomonas*.

On solid aniline-mineral agar the new isolate formed round, smooth, opaque colonies of 1 mm in diameter when grown at 30° C for 5 days. Colonies exhibited a characteristic yellow halo after 3 days of incubation on aniline-agar which disappeared during further incubation.

Cells were Gram-negative, polarly flagellated short rods. Oxidase and catalase: positive. β-Galactosidase, arginin-dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, and tryptophane desaminase activity could not be detected. Cells failed to liquify gelatine. Nitrate was reduced accompanied with the liberation of nitrogen. Hydrogensulfide was not generated from thiosulfate. No growth was observed on arabinose, mannitol, inositol, rhamnose, saccharose, melobiose, citrate, sorbitol, amygdaline, geraniol or on McConkey agar. Good growth was observed on acetate, nicotinate and pyruvate.

Strain JL1 grew well in liquid culture at the expense of 2MA as long as the concentration of the aromatic substrate did not exced 4 mM. Therefore during growth with 2MA the carbon source was added in portions and its concentration monitored by use of hplc analysis of the culture fluid. Under these conditions the maximum growth rate with 2MA as sole source of carbon was $\mu = 0.13\ h^{-1}$. The organism grew considerably faster with aniline (maximum growth rate $\mu = 1.5\ h^{-1}$) and tolerated higher substrate concentrations (≤ 15 mM).

Table 1. Specific activities of characteristic catabolic enzymes from aniline- and 2-methylaniline-grown cells of *Pseudomonas* sp. JL1[a]

Enzyme activity	Assays substrate	sp. act.[b] growth substrate	
		Aniline	2-Methyl-aniline
Catechol 1,2-dioxygenase	Catechol	22	1
	3-Chlorocatechol	1	1
Catechol 2,3-dioxygenase	Catechol	40	1070
	3-Methylcatechol	680	630
	4-Methylcatechol	290	360
2-Hydroxymuconic-semialdehyde dehydrogenase (HMSD)	2-Hydroxy-6-oxohexa-2,4-diienoate	230	190
	2-Hydroxy-5-methyl-6-oxo-hexa-2,4-dienoate	87	77
2-Hydroxymuconic-semialdehyde hydrolase (HMSH)	2-Hydroxy-6-oxo-hepta-2,4-dienoate	90	88

[a] Cells were harvested during exponential growth. Enzyme activities were determined as described in the text
[b] U/g Protein

Characteristic enzyme activities for aniline and 2-methylaniline (2MA) utilization

Specific enzyme activities in cell free extracts of aniline and 2MA grown cells (Table 1) clearly indicated that both substrates are converted to catechols which are subject to *meta*-cleavage. Catechol 1,2-dioxygenase (C120) activities were marginal when compared to catechol 2,3-dioxygenase (C230) which exhibited high activities not only with catechol but also with 3-methyl- and 4-methylcatechol. Correspondingly, typical enzyme activities of the *meta*-cleavage pathway such as 2-hydroxymuconic semialdehyde dehydrogenase (HMSD) and 2-hydromuconic semialdehyde hydrolase (HMSH) were detected in the cell free extracts. *meta*-Cleavage activity as the dominating mode of ring cleavage readily explains the bright yellow coloration ($\lambda_{max} = 375$ and 388 nm) of the culture fluid during growth with aniline or 2MA.

Turnover of anilines and chloroanilines

If the initial catabolic function of aniline and 2MA dioxygenation shall be recruited to take on a new role in chloroaniline degradation aniline or 2-methylaniline grown cells of strain JL1 must cooxidize chloroanilines yielding chlorocatechols as primary metabolites. The data listed in Table 2 indicate that during growth with aniline or 2MA

Table 2. Relative rates of oxygen-uptake at the expense of aniline and chlorosubstituted anilines by freshly harvested and washed cells of wild type and derived strains

Assay substrate	Relative rates of oxygen uptake[a] (strain, growth substrate)					
	JL1, aniline	JL1, 2-methyl-aniline	JL2, 3-chloro-aniline	JL3, 3-chloro-aniline	JL4, 3-chloro-aniline	JL5, 3-chloro-aniline
Aniline	100 (26)	100 (3)	100 (5)	100 (7)	100 (9)	100 (4)
2-Chloroaniline	25	26	64	92	69	137
3-Chloroaniline	10	ND	103	86	130	237
4-Chloroaniline	< 10	13	57	98	78	98

[a] Measurement of oxygen-uptake as described in "Materials and methods". Reactions rates are expressed as percentages of that for anilines (=100%). Absolute activities as micromol of O_2 per minute per ml of cell suspension (OD_{546} 1.0) are given in parenthesis
ND = not determined

an aniline dioxygenase was induced, which also cooxidized chloroanilines. The relative activities for differently substituted anilines were essentially the same for aniline or 2MA grown cells, indicating the same enzyme(s) are involved in the dioxygenation of both substrates.

Since absolute activities were considerably higher in aniline rather than 2MA grown cells, subsequent experiments on the evolution of chloroanilines degrading hybrids were carried out with aniline grown cells. In contrast to observations of other investigators (Reber et al. 1979; Schukat et al. 1983) chlorocatechols were not accumulated during chloroaniline cooxidation. Obviously O_2-uptake rates at the expense of chlorocatechols were always high compared to those observed with chloroaniline. The dioxygenation of chloroanilines was always slower than the ring cleavage of chlorocatechols, so that the latter metabolites did not accumulate.

Isolation of chloroanilines-utilizing strains

In order to isolate organisms, which are able to degrade and utilize chloroanilines as sole source of carbon and energy simple experimental procedures were applied in order to allow genetic exchange between *Pseudomonas* strain JL1 and *Pseudomonas* sp. B13 carrying complementary catabolic routes.

Initially both organisms were grown in a chemostat under prolonged selective conditions. The reservoir contained as growth substrate (5 mM each) 3-chlorobenzoate for strain B13 and aniline for strain JL1. In order to select chloroaniline degrading hybrid organisms 4-chloroaniline (4CA) was introduced into the reservoir at low concentration ($\leq$ 1 mM). Under continuous growth conditions the dilution rate was adjusted to $D \leq 0.016$ (h^{-1}). At higher dilution rates 4CA was accumulated in the culture fluid and the population washed out.

Even after prolonged selection in the chemostat over a period of approximately 6 weeks the generation of 4CA utilizing hybrids could not be detected by plating on mineral agar containing 4CA (0.5 mM) as sole carbon source. However, when cells from the chemostat were plated on aniline (5 mM) containing mineral agar a number of colonies were clearly distinguished from wild type cells of strain JL1. Colonies from the latter organism exhibited a characteristic yellow halo after 3 days of incubation on aniline agar. In contrast the new type of colonies orginating from a chemostat inoculum did not exhibit this property. Correspondingly batch cultures derived from these colonies also grew with aniline without generating the characteristic yellow colour of the original aniline degrader. These cultures harboured the ability to cooxidize all three isomeric chloroanilines at approximately the same rate as strain JL1. In the case of 3-chloroaniline, however, complete degradation and, on prolonged incubation, growth at the expense of this carbon source was observed. By streaking on a 3CA (2 mM) mineral agar a pure culture (*Pseudomonas* strain JL2) was obtained capable of growth on 3CA. The nutritional properties of strain JL2 are incorporated in Table 3 and clearly indicate that this organism originates from the parent strain B13 which must have acquired the genes for aniline dioxygenation. Obviously the catabolic functions of the meta-cleavage pathway were not cotransferred so that the hybrid strains could not utilize methylanilines.

Table 3. Nutritional properties of wild-types and derived strains

Assay substrate	*Pseudomonas* sp.					
	B13	JL1	JL2	JL3	JL4	JL5
Aniline	−	+	+	+	+	+
2-Methylaniline	−	+	−	−	−	−
3-Methylaniline	−	+	−	−	−	−
4-Methylaniline	−	+	−	−	−	−
2-Chloroaniline	−	−	−	−	+	−
3-Chloroaniline	−	−	+	+	+	+
4-Chloroaniline	−	−	−	+	+	−
Acetate	+	+	+	+	+	+
Nicotinic acid	−	+	−	ND	ND	+
Geraniol	+	−	+	ND	ND	−
McConkey agar	+	−	+	ND	ND	−

Nutritional properties were determined on mineral salts agar containing the assay substrate as sole source of carbon and energy (see Materials and methods)
Symbols: + = good growth, − = no growth after 14 days, ND = not determined

Population analysis revealed that all colonies exhibiting colourless growth with aniline were able to grow with 3CA but were unable to utilize the isomeric chloroanilines.

When strain JL2 was grown in batch culture on 3CA in the presence of small amounts of 4CA ($\leq$ 0.5 mM) within 3 weeks slow growth at the expense of the new carbon source was observed. On subcultivation in batch culture and finally on 4CA-agar (2 mM) a pure culture (strain JL3) was isolated. The 4CA acclimated derivative strain still harboured the capability of growth with 3CA.

The new isolate could also be adapted to the utilization of 2CA, when being exposed to low concentrations ($\leq$ 2 mM) of this substrate. Compared to the immediate onset of growth with 3CA and 4CA as substrates a long lag period is required for the utilization of 2CA.

By comparison of the characteristic nutritional properties of the hybrid strains with those of their parent organisms it became evident that the 3CA degrader from the chemostat enrichment, strain JL2, and consequently the derivative strains JL3 and JL4 had the genetic background of *Pseudomonas* sp. B13. Obviously these organisms have acquired the capability of aniline and chloroaniline dioxygenation. Consequently these compounds were new growth substrates for B13. The catabolic functions of the *meta*-cleavage pathway from the donor strain JL1 were not cotransferred, so that the transconjugants did not acquire the ability to utilize methylanilines. For the same reason the B13 derivative strains (in contrast to the donor) utilized aniline via *ortho*-cleavage.

The 3CA utilizing strain JL5 which has been obtained by conjugation on solid medium resembles the parent strain JL1. Mating experiments under varying conditions revealed that 3CA must be present as a selective substrate during conjugation. When cell suspensions of both parent strains were incubated on nutrient agar or on 3CB-aniline agar in the absence of 3CA no transconjugants could be isolated. Strain JL5 has completely lost the ability to utilize methylanilines. Nevertheless the nutritional properties indicate the transfer of genes from *Pseudomonas* sp. B13 necessary for the degradation of chlorocatechols to strain JL1 as the recipient organism.

Growth on chloroanilines in batch culture

The chlorosubstituted anilines listed in Table 4 were used as growth substrates in liquid media. Because of the toxicity of chloroanilines substrate concentrations were kept below 4 mM. Under these conditions the cultures grew exponentially with maximum growth rates listed in Table 4. HPLC-analysis and chloride determination in the culture fluid indicated that substrate comsumption were clearly correlated to the release of equimolar quantities of chloride.

Oxidation of chloroanilines

Compared to the parent strain JL1 relative initial activities of oxidation of chlorosubstituted anilines were considerably higher in all transconjugants irrespective of the growth substrate used (see Table 2).

Activities of catabolic enzymes in cell-free extracts

Typical enzyme activities for productive break down of the halosubstituted aromatic ring of chloroanilines were induced in cells grown on 3-chloroaniline. The latter compound is a common growth substrate for all the hybrid strains so that comparable kinetic data became available (see Table 5). Catechol 1,2-dioxygenase (C120) with high activities for chlorosubstituted catechols, cycloisomerase activity for 2-chloro-*cis*, *cis*-muconate and hydrolase activity for 4-carboxymethylenebut-2-en-4-olide were induced and corresponded to the catabolic activities found in the parent strain B13 during growth on 3-chlorobenzoate. As far as 4CA and 2CA served as growth substrates, the specific and relative activities of these enzymes were similar to those in 3CA utilizing cells. *meta*-Cleavage activity was not observed under any conditions of growth.

Table 4. Doubling-times (h) during growth of the derive strains on chloroanilines as sole source of carbon and energy

Substrate	Bacterial strains			
	JL2	JL3	JL4	JL5
2-Chloroaniline	–	–	~20	–
3-Chloroaniline	5.5	12.5	7	11
4-Chloroaniline	–	23	15	–

The organisms were grown in 1 l-fermentor as described in Materials and methods

Regioselectivity of aniline dioxygenation

2CA can be subject to 1,2- or 1,6-dioxygenation. The primary attack in 1,2-position would eliminate both ammonia and chloride, whereas the alternative mode of hydroxylation would generate 3-chlorocatechol (see Fig. 1). Correspondingly the two alternative modes of dioxygenation would generate 3- and 4-chlorocatechol from 3CA. In contrast, only 4-chlorocatechol can be generated from 4CA dioxygenation.

Methylanilines as structural analogues of chloroanilines were readily cooxidized by chloroaniline grown cells of strain JL2 yielding methylsubstituted 4-carboxymethyl-but-

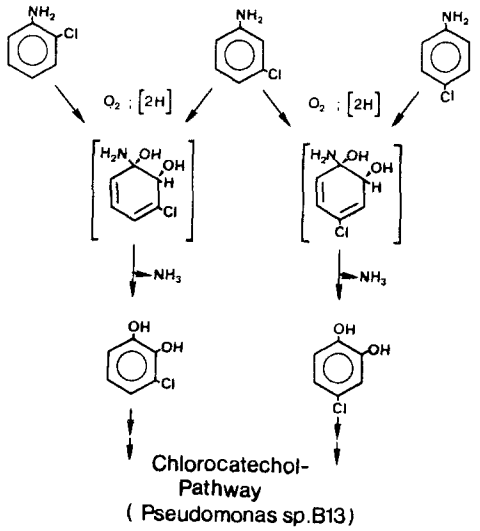

Fig. 1. Initial reactions of chloroaniline degradation

Table 5. Specific activities of catabolic enzymes in cell-free extracts of 3-chloroaniline-grown cells of *Pseudomonas* sp. JL2 – JL5[a]

Activity	Assay substrate	sp. act. (U/g protein) Strain *Pseudomonas* sp.			
		JL2	JL3	JL4	JL5
Catechol 1,2-dioxygenase	Catechol	220 (100)	430 (100)	310 (100)	140 (100)
	3-Chlorocatechol	(123)	(137)	(129)	(117)
	4-Chlorocatechol	(100)	(105)	(94)	(91)
	3,5-Dichlorocatechol	(96)	(97)	(81)	(60)
Catechol 2,3-dioxygenase	Catechol	< 1	< 1	< 1	< 1
Muconatecycloisomerase	*cis*,*cis*-Muconate	23	19	ND	6
	2-Chloro-*cis*,*cis*-muconate	42	43	40	12
Carboxymethylenebut-2-en-4-olide-hydrolase	4-Carboxymethylene-but-2-en-4-olide	1570	1200	1180	1420

[a] For preparation of cell free extracts cells were harvested during exponential growth. Enzyme activities were determined as described in the text and are expressed as absolute specific activities (micromol per minute per gram of protein). Relative activities of catechol 1,2-dioxygenase (given in parenthesis) are referred to catechhol as 100%

2-en-4-olides as dead end metabolites (Hartmann et al. 1979). HPLC analysis revealed that 4-carboxymethyl-4-methylbut-2-en-4-olide was the only metabolite from 2-methylaniline as well as from 3-methylaniline. This indicates that dioxygenation of 2- and 3-substitutet anilines must be highly regioselective generating 3-chlorocatechol from 2CA through 1,6-dioxygenation and from 3CA through 1,2-dioxygenation. 4-Carboxymethyl-2-methylbut-2-en-4-olide was the metabolite from cooxidation of 4-methylaniline.

Discussion

Haloaromatics degrading bacteria of several genera have been isolated from different habitats and parts of the world. Conventional enrichment techniques can be applied as long as chlorinated substrates like mono- or dichlorophenoxyacetic acids or 3-chlorobenzoate were used as a selective substrate (Tiedje et al. 1969; Evans et al. 1971; Fisher et al. 1978; Kilpi et al. 1980). These compounds have little inherent toxicity when compared to other chloroaromatics such as chlorobenzene, chlorophenols or chloroanilines, which are of practical relevance in industrial waste streams. The isolation of haloaromatics degrading organisms from very different places of the world by different investigators together with recent observations from this laboratory (Knackmuss 1983) indicate that the crucial catabolic sequence of halocatechol assimilation preexists in nature and lurks in a few marginal members of the indigenous microflora. Other investigators have shown that the capability of aniline degradation and chloroaniline cooxidation is also present in nature (Reber et al. 1979; Surovtseva et al. 1980); You and Bartha 1982). Therefore, one could expect that the compound potential of chlorocatechol generation plus chlorocatechol assimilation may also preexist in a single organism or may readily be assembled during enrichment with chloroanilines as substrates of selection (see Zeyer and Kearney 1982).

High inherent toxicities or inhibiting effects of chloroanilines and/or chlorocatechols, generated through cometabolic activities, may suppress the enrichment of marginal members of the indigenous communities or the assembly of new hybrid pathways during the conventional enrichment process.

The present paper clearly demonstrates that in principle these existing complementary catabolic sequences can be put together to take on a new role in chloroaniline degradation. Obviously, when exposure to chloroanilines as new selective substrates is carefully controlled by growing the complementary strains in a chemostat or on solid media, exchange of genetic information and evolution of new pathways for total degradation and utilization of chloroanilines can be accelerated.

We have recently reported the construction of new pathways for the degradation of simple chloroaromatics such as chlorobenzoates, chlorosalicylates and chlorophenols by making use of overlap of existing complementary catabolic sequences. These experiments have shown that on one hand the functionality of *Pseudomonas* sp. B13 towards a broader spectrum of chloroaromatics can be amplified through the acquisition of genetic information (e.g. part of the TOL plasmid) for chlorocatechol generating sequences (Reineke and Knackmuss 1979, 1980). On the other hand we have shown that the genes encoding chlorocatechol assimilation of *Pseudomonas* sp. 13 are themselves transmissible (Reineke et al. 1982b). Thus an *Alcaligenes* strain with unusual resistence to higher concentration of phenol could readily be converted to a chlorophenol degrader with improved degradative capacity (Schwien and Schmidt 1982). Correspondingly a functional hybrid pathway for chloroanilines (see Fig. 1) could evolve in *Pseudomonas* sp. B13 through the acquisition of genes encoding aniline dioxygenation or in the aniline degrading strain JL1, if strain B13 could donate its genetic information for chlorocatechol assimilation. The outcome of the mating experiments indicate that exchange of genetic material is feasable in both directions. Apparently the chloroaniline degrading transconjugant JL5 has been selected because strain JL1 could acquire the chlorocatechol degrading capability from *Pseudomonas* sp. B13, which includes at least four enzymes, a catechol 1,2-dioxygenase (pyrocatechase II), a muconate cycloisomerase II and a 4-carboxymethylene-but-2-en-4-olide hydrolase (Dorn and Knackmuss 1978b; Schmidt and Knackmuss 1980) and a maleylacetate reductase (Reineke, unpublished results). Under the conditions of the continuous culture strain B13 could also acquire a genetic information from strain JL1, which enabled the transconjugants JL2, JL3, and JL4 to utilize aniline and consequently also chloroanilines. However, none of the chloroaniline degrading transconjugants are capable of growth on methylanilines. This adaptive response is due to the incompatibility of chloro- and methylsubstituted aromatic compounds as growth substrates. The *meta*-cleavage appears to be unproductive for the breakdown of haloaromatics, so that during selection with chloroaromatics total suppression of *meta*-cleavage (by genetic inactivation) must occur (see Knackmuss 1983).

Obviously the evolution of a new pathway for chloroaniline degradation through the combination of previously separate catabolic routes must involve a number of additional genetic events, such as changes in the regulation, specificity and activity of the aniline dioxygenase and in the prevention of the nonproductive *meta*-cleavage pathway (originally present in the parent strains JL1). A similar evolutionary process has been analyzed in more detail during the acquisition of parts of the TOL plasmid by *Pseudomonas* sp. B13 (Reineke et al. 1982a)

The conditions under which these hybrid pathways for chloroaniline degradation evolved clearly indicates that in an industrial sewage treatment system the establishment of a chloroaniline degrading activity may be accomplished merely through the enhancement of the size of certain indigenous populations. For productive breakdown of chloroanilines this may be accomplished firstly by stimulating the chloroaniline cometabolizing population through the introduction of readily degradable aniline or toluidines. Secondly, the chlorocatechol degrading potential may be amplified by feeding nontoxic haloaromatics such as chlorophenoxyacetates or 3-chlorobenzoate. Since the genetic information for the productive breakdown of the latter substrates may reside in a few marginal members of the indigenous microflora evolution of a chloroaniline degrading activity in sewage treatment systems may be enhanced through the introduction of specially adapted laboratory strains such as *Pseudomonas* sp. B13 or the chloroaniline degraders described above.

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