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The in vivo construction of 4-chloro-2-nitrophenol assimilatory bacteria

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Abstract. *Pseudomonas* sp. N31 was isolated from soil using 3-nitrophenol and succinate as sole source of nitrogen and carbon respectively. The strain expresses a nitrophenol oxygenase and can use either 2-nitrophenol or 4-chloro-2-nitrophenol as a source of nitrogen, eliminating nitrite, and accumulating catechol and 4-chlorocatechol, respectively. The catechols were not degraded further. Strains which are able to utilize 4-chloro-2-nitrophenol as a sole source of carbon and nitrogen were constructed by transfer of the haloaromatic degrading sequences from either *Pseudomonas* sp. B13 or *Alcaligenes eutrophus* JMP134 (pJP4) to strain N31. Transconjugant strains constructed using JMP134 as the donor strain grew on 3-chlorobenzoate but not on 2,4-dichlorophenoxyacetate. This was due to the non-induction of 2,4-dichlorophenoxyacetate monooxygenase and 2,4-dichlorophenol hydroxylase. Transfer of the plasmid from the 2,4-dichlorophenoxyacetate negative transconjugant strains to a cured strain of JMP134 resulted in strains which also had the same phenotype. This indicates that a mutation has occurred in pJP4 to prevent the expression of 2,4-dichlorophenoxyacetate monooxygenase and 2,4-dichlorophenol hydroxylase.

Key words: Nitrite elimination – 4-Chlorocatechol metabolism – 2,4-Dichlorophenoxyacetic acid – 3-Chlorobenzoic acid

Nitro- and chloronitroaromatic compounds are highly toxic to all organisms, their toxicity against man and animals being attributed to their enzymatic reduction to nitroso and hydroxylamino compounds. These latter compounds may lead to the formation of either methaemoglobin, which is unable to bind oxygen, or of nitrosamines, which are carcinogenic (Wirth et al. 1971; Venulet and Van Etten 1970).

A well known property of nitrophenols is their uncoupling effect on electron transport phosphorylation (Simon 1953) and this has led to their use as herbicides. 2,4-Dinitrophenol was used also as a slimming agent in man which resulted in cases of blindness (Deutsche Apotheker Zeitung 1963).

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Both nitro- and chloronitroaromatic compounds are of considerable technical importance and are used as solvents and synthetic intermediates in the dyestuff and explosive industries. Their widespread use in industry and subsequent appearance in waste effluents has been the cause of failures in sewage treatment plants, contamination of rivers and of the atmosphere. A particular problem has arisen in areas in which residues have been found from factories manufacturing explosives during World War II. Such areas are highly contaminated with nitrotoluenes, nitrocresols and their reduction products and this has caused problems with drinking water due to such compounds entering the ground waters (Haas and v. Löw 1986).

Although there are several reports on the degradation of mono-nitrobenzoates (Cain 1958, 1966; Ke et al. 1959) and mono-nitrophenols (Raymond and Alexander 1971; Spain et al. 1979; Zeyer and Kearney 1984; Zeyer et al. 1986), comparatively little is known about the total degradation of di- and higher substituted nitroaromatic compounds (Germanier and Wuhrmann 1963; Jensen and Lautrup-Larsen 1967) and there have been few reports on the degradation of chloronitroaromatic compounds (Teuteberg 1964; Murthy and Kaufmann 1978). The complete mineralization of the latter compounds appear to be rare in nature, however partial degradation which is accompanied by the release of nitrite is obviously widespread. Therefore it seems necessary to combine the metabolic capabilities from different bacterial species to effect the complete mineralization of such xenobiotics.

Bruhn et al. (1987) have described the isolation of bacteria which are able to eliminate and assimilate the nitro group of some nitroaromatic compounds in the presence of a readily degradable carbon source. The elimination of the nitro group from chloronitroaromatic compounds leads to the formation of chlorohydroxycompounds such as chlorophenols and chlorocatechols, whose further degradation is dependent upon the action of haloaromatic degrading enzymes to mineralize them to CO_2 and H_2O. *Pseudomonas* sp. B13 (Dorn et al. 1974) and *Alcaligenes eutrophus* JMP134 (Pemberton et al. 1979) synthesize haloaromatic degrading enzymes and there have been reports on the use of *Pseudomonas* sp. B13 to construct strains which degrade such compounds as chloroanilines (Latorre et al. 1984), and chlorosalicylates (Rubio et al. 1986). Don and Pemberton (1981) and Friedrich et al. (1983) have shown that the genes encoding the haloaromatic enzymes in *Alcaligenes eutrophus* JMP134, which are encoded on the plasmid pJP4, can be

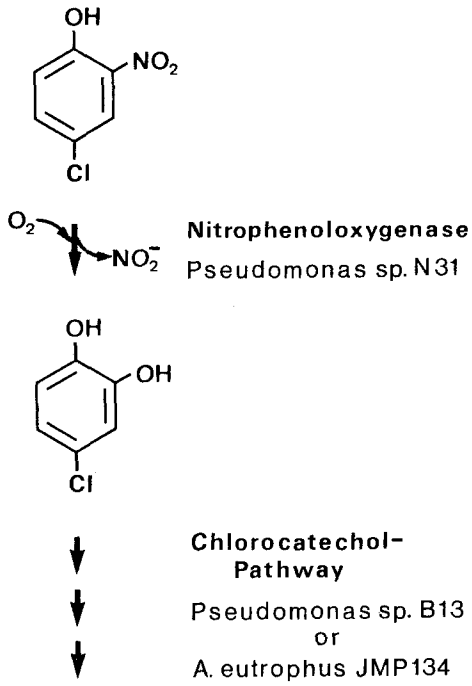

Fig. 1. Degradation of 4-chloro-2-nitrophenol by in vivo constructed strains of *Pseudomonas*

transferred to, and expressed in, several different Gram-negative species.

The present communication describes the isolation of a *Pseudomonas* sp. which is able to use 4-chloro-2-nitrophenol (4C2NP) as sole source of nitrogen accumulating 4-chlorocatechol and the in vivo construction of strains which can utilize 4C2NP as a source of nitrogen, carbon and energy. This is accomplished by transfer of the haloaromatic degrading sequences of *Pseudomonas* sp. B13 or *A. eutrophus* JMP134, into the newly isolated strain, designated *Pseudomonas* sp. N31 (Fig. 1).

Materials and methods

Organisms

The bacterial strains used in this study together with their relevant phenotypic properties, are listed in Table 1. *Pseudomonas* sp. strain N31 was isolated from soil samples from Göttingen (FRG) using 3-nitrophenol (3NP) and succinate as sole sources of nitrogen and carbon, respectively. Mixed soil samples were inoculated into 50 ml nitrogen-free mineral medium (Bruhn et al. 1987) supplemented with 0.5 mM 3NP and 20 mM succinate. After incubation at 30° C on a rotary shaker for 2–3 days, 5 ml was transferred into the same medium and, after incubation for 2 days, the culture was plated onto 3NP-succinate agar plates. Single colonies were subcultivated on the same medium and one of them, designated N31, was selected for further study. Strain N31 was identified as a *Pseudomonas* sp. (G. Auling, unpublished work). *Alcaligenes eutrophus* JMP134-1 is a derivative of JMP134 (Don and Pemberton 1981) which expresses constitutively all the enzymes required for the degradation of 2,4-dichlorophenoxyacetic acid. This strain was isolated by Pieper et al. (unpublished work).

Media and culture conditions

For testing the growth of strains with a nitrophenol as sole nitrogen source, the mineral medium described previously (Bruhn et al. 1987) was used with 0.5 mM of the nitroaromatic compound and with 20 mM of succinate as carbon source. To test for the use of a nitrophenol as a sole source of nitogen and carbon, the nitrophenol concentration was increased to 1 mM and succinate was omitted. *Pseudomonas* sp. B13-50 and *A. eutrophus* strains JMP134 and 134-1 were grown in the mineral medium described by Dorn et al. (1974) using 2.5 mM 3-chlorobenzoate (3CB) and 2.5 mM 2,4-dichlorophenoxyacetate (24D) as carbon sources, respectively.

Solid media were prepared by the addition of 1.5% (w/v) oxoid agar No. 1 (Oxoid Ltd., London, England). Where antibiotics were used they were added to media as sterile solutions at the concentrations shown in Table 1.

Preparation of cell-free extracts and cells for enzyme assays

For the determination of the activity of enzymes in the degradation of haloaromatic compounds, cells were grown in the mineral medium described by Dorn et al. (1974) with 20 mM acetate and, as inducers, 4C2NP (1 mM), 3CB (2.5 mM) or 24D (2.5 mM). After overnight incubation additional inducer (0.2 mM) was added to the culture and incubation continued for 2 h, when the cells were harvested and resuspended in 50 mM Tris-hydrochloride buffer, pH 7.0, which contained 2 mM $MnCl_2$. Cell extracts were prepared as described by Latorre et al. (1984). Cultures of *Pseudomonas* sp. strain N31 used to detect enzymes responsible for the elimination of nitrite from nitroaromatic compounds, were treated as described by Spain et al. (1979).

Enzyme assays

Catchol 1,2-dioxygenase (EC 1.13.11.1) was assayed by the method of Dorn and Knackmuss (1978), muconate cycloisomerase (EC 5.5.1.1) and 4-carboxymethylenebut-2-en-4-olide (dienelactone) hydrolase were tested by the method of Schmidt and Knackmuss (1980). 2,4-Dichlorophenoxyacetic acid monooxygenase and 2,4-dichlorophenol hydroxylase were assayed by determining oxygen uptake by washed whole cells in a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH, USA). Nitrophenol oxygenase was assayed essentially as described by Zeyer et al. (1986) except that the concentration of NADPH was 2 mM. Assays were carried out within 3–4 h of the preparation of the cell extracts. One unit of enzyme activity was defined as the amount of enzyme necessary to produce or convert 1 μmol of product or substrate, respectively, per min. Specific activities were expressed as units per gram of protein. Protein content of the extracts was determined by the method of Bradford (1976).

Turnover of 4-chloro-2-nitrophenol by resting cells of Pseudomonas sp. strain N31

The organism was grown with 0.5 mM 4C2NP as sole source of nitrogen and 20 mM succinate in the mineral medium described previously (Bruhn et al. 1987), and resuspended in 20 mM phosphate buffer (optical density at 546 nm, 2.5–3). The cell suspensions were incubated at 30° C on a

Table 1. Bacterial strains

Strain	Relevant phenotype	Derivation/reference
Pseudomonas spp.		
N31	$3NP^+$, $4C2NP^-$, $3CB^-$, $24D^-$, Sm^s	This paper
N31-53	As N31 but Sm^r	Spontaneous selection on NBA/Sm25
N31-54	As N31 but Rp^r	Spontaneous selection on NBA/Rp100
B13 (DSM426)	$3NP^-$, $4C2NP^-$, $3CB^+$, Sm^s	Dorn et al. 1974
B13-50	As B13 but Rp^r	Spontaneous selection on NBA/Rp50
N31-1	$3NP^+$, $4C2NP^+$, $3CB^+$	B13 × N31, selection on 4C2NP
N31-57	$3NP^+$, $4C2NP^+$, $3CB^+$, $24D^-$, Sm^r, Hg^r	JMP134 × N31-53, selection on 4C2NP/Sm25 (see text)
N31-58	AsN31-57	JMP134-1 × N31-53, selection as for N31-57
N31-59	$3NP^+$, $4C2NP^+$, $3CB^+$, $24D^-$, Rp^r, Hg^r	JMP134-1 × N31-54, selection on 4C2NP/Rp100
N31-60	AsN31-59	JMP134-1 × N31-54, selection as for N31-59
N31-61	As N31-57, but Hg^s	B13-50 × N31-53, selection as for N31-57
Alcaligenes eutrophus		
JMP134	$3CB^+$, $24D^+$, Hg^r, Sm^s	Pemberton et al., 1979
JMP134-1	AS JMP134	Pieper et al., unpublished work
JMP222	$3CB^-$, $24D^-$, Hg^s, Sm^r	Don and Pemberton, 1981
JMP289	$3CB^-$, $24D^-$, Hg^s, Rp^r	Don and Pemberton, 1981

3NP, 3-nitrophenol; 4C2NP, 4-chloro-2-nitrophenol; 3CB, 3-chlorobenzoate, 24D, 2,4D-dichlorophenoxyacetate; Sm, streptomycin; Rp, rifampicin; Hg^r, resistant to inorganic mercuric ions; NBA/Sm25, nutrient broth agar/streptomycin 25 μg ml^{-1}; NBA/Rp50, nutrient broth agar/rifampicin 50 μg ml^{-1}
DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, FRG

water bath shaker (New Brunswick, Edison, NJ, USA) and 0.5 mM 4C2NP and 1 mM $(NH_4)_2SO_4$ were added; the latter was added to prevent the assimilation of the nitrite released by the organism.

Analysis of substrates and metabolites

4-Chloro-2-nitrophenol, nitrite and 4-chlorocatechol in the culture fluid were detected and quantified by reverse-phase high-pressure liquid chromatography (HPLC) as described previously (Bruhn et al. 1987). Additionally, nitrite was estimated colorimetrically (Montgomery and Dymoc 1961).

Chemicals

2- and 3-Nitrophenol, 4-chloro-2-nitrophenol, 3-chlorobenzoic acid and succinate were obtained from Fluka, Buchs, Switzerland. Catechol was purchased from E. Merck AG, Darmstadt, FRG and 2,4-dichlorophenoxyacetic acid was from Aldrich-Chemie, Steinheim, FRG.

3-Chloro- and 4-chlorocatechol were prepared as described previously (Latorre et al. 1984). *cis,cis*-Muconic acid was prepared by the method of Wacek and Fiedler (1949) and 2-chloromuconic acid and 4-carboxymethylenebut-2-en-4-olide were prepared as described by Schmidt et al. (1980). 2,4-Dichloro-*cis,cis*-muconic acid was prepared from 3,5-dichlorocatechol using a semi-purified preparation of catechol 1,2-dioxygenase, free of cycloisomerase, from 2,4-dichlorophenoxyacetate-grown cells of *Alcaligenes eutrophus* JMP134.

Results

Properties of Pseudomonas sp. strain N31

Some of the nutritional properties of N31 are shown in Table 2. Growth occurred in succinate medium which contained either 2NP, 3NP or 4C2NP as sole source of nitrogen, but only 3NP could be used as a sole source of both carbon and nitrogen (Fritz 1987).

In the case of 2NP/succinate and 4C2NP/succinate the yellow color of the medium turned black which may have been due to the formation and subsequent autoxidation of catechol and 4-chlorocatechol (4CC), respectively. The metabolism of 3NP did not lead to the elimination of nitrite, therefore ammonia could be detected (Fritz 1987). The degradation of 3NP is the subject of further investigations.

Turnover of 4-chloro-2-nitrophenol by resting cells of Pseudomonas sp. strain N31

After incubation with resting cells of *Pseudomonas* sp. N31, 4C2NP was completely converted, nitrite was released stoichiometrically, but only 65% of the corresponding 4CC could be detected. After an incubation period of two hours the color of the medium changed from yellow to brown and finally to black, indicating the autoxidation of 4CC.

Construction of hybrid strains of Pseudomonas sp. B13 and Alcaligenes eutrophus strains JMP134 and JMP134-1 with Pseudomonas sp. N31

The donor strains, B13-50, JMP134 and 134-1, were grown to exponential phase in nutrient broth (NB) and 500 μl of

Table 2. Phenotypic properties of wild types and derivative strains[a]

Substrate	*Pseudomonas* spp					*Alcaligenes eutrophus* JMP134
	N31	B13-50	N31-1	N31-57	N31-61	
2-Nitrophenol	−	−	+	+	+	−
2-Nitrophenol/Succinate	+	−	+	+	+	−
3-Nitrophenol (0.5 mM)	+	−	+	+	+	+
3-Nitrophenol/Succinate	+	−	+	+	+	+
4-Chloro-2-nitrophenol (1 mM)	−	−	+	+	+	−
4-Chloro-2-nitrophenol/Succinate	+[b]	−	+	+	+	−
3-Chlorobenzoate (5 mM)	−	+	+[c]	+[c]	+[c]	+
2,4-Dichlorophenoxyacetate (2.5 mM)	−	−	−	−	−	+
Benzoate (5 mM)	+[b]	+	+[b]	+[b]	+[b]	+
Sebacic acid (5 mM)	−	+	−	−	−	+
Mannose (5 mM)	+	−	+	+	+	−
Ribose (5 mM)	+	−	+	+	+	−
Geraniol (vapour)	−	+	−	−	−	−
Nutrient broth agar 41°C	−	+	−	−	−	n.d.

[a] The growth was tested on agar plates except that the growth with 2NP and 3NP as sole source of carbon and energy was tested in liquid culture. The composition of the nitrophenol/succinate medium see Material and methods

[b] After 2–3 days incubation, the medium turned black

[c] Growth occurred after incubation for 5 days

Note: Strains N31-58, 59 and 60 had the same phenotype as N31-57; JMP134-1 has the same phenotype as JMP134

each culture was mixed, separately, with 500 µl of an exponential phase NB culture of an appropriate antibiotic resistant strain of *Pseudomonas* N31 (Table 1). The mixtures were centrifuged for 1 min at 4000 rpm, most of the supernatant fluid was discarded and the concentrated, mixed cell suspension transferred to a 0.2 µm Millipore filter (Millipore, Eschborn, FRG) on a NB agar plate. After incubation overnight at 30°C, the cells which had been grown on the filter were resuspended in 1 ml saline (9 g NaCl and 0.1 g $MgSO_4 \cdot 7\ H_2O$ per l), and the cell suspension plated at varying dilutions on mineral agar plates containing the relevant antibiotic and 1 mM 4C2NP as sole source of carbon and nitrogen. Similarly, strain B13 was conjugated with *Pseudomonas* N31 and transconjugants selected on 4C2NP as the sole source of carbon and nitrogen. Transconjugant colonies appeared after 8–10 days incubation. Several colonies were purified from each sample and their phenotypes determined on the substrates shown in Table 2. One colony was selected at random from each conjugation experiment and designated as shown in Table 1. The frequency of isolation of transconjugants which could use 4C2NP as sole source of carbon and nitrogen was approximately 10^{-6} per donor cell in each case (data not shown).

The formation of hydrid strains from B13 and N31 could also be demonstrated by streaking the parental strains together on 4C2NP agar plates. The two strains were grown on NB agar and a heavy inoculum of both strains was applied to the same area of a 4C2NP agar plate. After incubation of 30°C for 7 days, colonies were observed in the area where the two strains were mixed and, in this area, the yellow color of the medium disappeared. To ensure that the two strains were not acting syntrophically, they were also plated out parallel to one another (approximately 2 mm apart) on 4C2NP agar; no growth was observed after 14 days incubation.

Phenotypic and nutritional properties of transconjugant strains

Each of the transconjugant strains N31-1, N31-57, 58, 59, 60 and 61 (Table 1) had the same phenotypic properties when tested as shown in Table 2. With the exception of their ability to utilize 4C2NP as a sole source of carbon and nitrogen and their growth on 3-chlorobenzoate, the transconjugants had the same properties as the recipient strain, *Pseudomonas* sp. N31. Growth of both N31 and the transconjugant strains on benzoate, resulted in the formation of a black color in the medium and this was presumed to be due to the accumulation of catechol which undergoes autoxidation. Donor strains, *Pseudomonas* sp. B13-50 *Alcaligenes eutrophus* JMP134 and 134-1 utilize benzoate without the accumulation of catechol. All the transconjugant strains grew slowly on 3CB but unexpectedly, transconjugant strains in which JMP134 and 134-1 were donors did not grow on 24D.

Enzymatic activities of the parental and transconjugant strains

The specific activities of the donor, recipient and transconjugant strains under various induction conditions are shown in Table 3. From the data it is clear that the transconjugant strains carry the genes encoding the enzymes required for the degradation of 4-chlorocatechol which would be formed by the action of the nitrophenol oxygenase on 4-chloro-2-nitrophenol. In each of the transconjugants tested, the level of catechol 1,2-dioxygenase (C120) and muconate cycloisomerase activity was always significantly less than that in the donor strains when the same inducer was used. The level of expression of these enzymes was approximately the same when 4C2NP was used as an inducer as when either 3CB (for N31-61) or 24D (for N31-57 and N31-58) was used. The ability of 4C2NP to act as an inducer in B13-50, JMP134 or JMP134-1 of any of the enzymes

Table 3. Specific activities (U/g protein) of characteristic catabolic enzymes of wild type and derivative strains[a]

Enzyme activity	Substrate	Strains/inducers							
		N31 4C2NP	B13 3CB	N31-1 4C2NP	N31-61 3CB	N31-61 4C2NP	JMP134 24D	N31-57 24D	N31-57 4C2NP
Nitrophenol oxygenase	2-Nitrophenol	60	<1	NT[b]	<1	63	<1	<1	74
Catechol 1,2-oxygenase (C120)	Catechol	<1	1080	118	28	29	164	65	74
	3-Chlorocatechol	<1	265	160	23	29	222	69	74
	4-Chlorocatechol	<1	310	120	27	32	201	88	99
Muconate cycloisomerase	*cis,cis*-Muconate	<5	108	8	16	18	<5[c]	<5	13
	2-Chloro-*cis,cis*-muconate	<5	350	25	8	<5	<5[c]	<5	<5
	2,4-Dichloro-*cis,cis*-muconate	<5	NT	NT	8	63	240[c]	53	274
Carboxymethylenebut-2-en-4-olide hydrolase	trans-4-Carboxymethylene-but-2-en-4-olide	<5	3660	5620	3220	6370	910	295	2520

[a] Strains were grown as described in Materials and methods
[b] NT not tested
[c] Data from Pieper et al. 1988
Note: Strain N31-58, a transconjugant of JMP134-1 × N31-53 showed similar activities to N31-57. Strain JMP134-1 had similar activities to JMP134. Strains N31-59 and N31-60 were not assayed for the above enzymes

assayed (Table 3), could not be determined as no growth occurred in the presence of 1 mM 4C2NP. The comparative levels of activity of muconate cycloisomerase and dienelactone hydrolase in the donor and transconjugant strains varied in different induction experiments (data not shown); the reason for this variation is not known and was not investigated further. Induction of the transconjugant strains N31-57, 58, 59 and 60 with either 24D or 3CB (1 mM) did not result in the synthesis of either 24D-monooxygenase or 2,4-dichlorophenol hydroxylase; both enzymes were induced when JMP134 was grown under the same conditions (data not shown).

Conjugation of transconjugant strains N31-57, 58, 59 and 60 with plasmid free strains of A. eutrophus JMP134

To investigate if the inability of N31-57 and 58 to grow on 24D was due to non-expression of either 24D monooxygenase and/or 2,4-dichlorophenol hydroxylase in the N31 background, each strain was conjugated to JMP289, a cured strain of JMP134, using 3CB and 24D (both in combination with rifampicin) as selective carbon sources. Similarly N31-59 and N31-60 were conjugated to JMP222 and selected on 3CB and 24D agar plates containing streptomycin. In each case transconjugants were obtained on 3CB but not 24D and all were Hg^r. Conjugation of JMP134 with either JMP222 or JMP289 gave transconjugants which grew on both 24D and 3CB.

Stability of the 4C2NP phenotype of the transconjugant strains

The stability of the phenotypes was tested after growth in nutrient broth (NB) and also in NB in the presence of mitomycin C. Strains were grown overnight and transferred on three successive days, using 100 µl inoculum to 10 ml NB. The cultures were diluted and plated to NB agar and approximately 250 colonies were tested for growth on 4C2NP, 3CB, 4C2NP/succinate and succinate agar plates. The procedure was repeated using NB plus 0.8 µg ml^{-1} mitomycin C. No significant loss of the 3CB phenotype of strains B13-50, JMP134 and JMP134-1 was found either after growth in NB or in the presence of mitomycin C. Growth of the transconjugant strain N31-61 in NB resulted in no significant loss of either the 4C2NP or 3CB phenotype. However, when mitomycin C was present, approximately 75% of the colonies tested were 4C2NP and 3CB negative, but all retained the ability to grow on 4C2NP/succinate. This showed that the loss of 4C2NP phenotype was due to the loss of the genes encoding the haloaromatic enzymes and not to the loss of the gene(s) encoding nitrophenol oxygenase.

When strains N31-57 and N31-58 were tested, approximately 50% of the colonies tested after growth in NB had lost both the 4C2NP and 3CB phenotype and this loss was increased to almost 100% when mitomycin C was present during growth. As before, all colonies retained the ability to grow on 4C2NP/succinate.

Discussion

Although microbes have the capacity to cometabolize many xenobiotic structures, complete pathways for the mineralisation and utilization of Man-made structures appear to be rare in nature. Consequently, ordinary enrichment techniques may fail if xenobiotic compounds are used as sole source of carbon and energy. Even continuous enrichment procedures may require prolonged incubation periods if multiple genetic changes are necessary to evolve an entire new pathway. A powerful strategy for the evolution of a new complete catabolic pathway is the in vivo assembly of partial catabolic sequences from different pathways and different organisms.

A case in point is 4C2NP which is too toxic for bacteria to be used as sole source of carbon and energy for conventional

enrichment procedures. Bruhn et al. (1987) however, reported the isolation of bacteria which could partially degrade nitroaromatic compounds as sole source of nitrogen at low substrate concentrations in the presence of a readily degradable carbon source. Using this strategy, *Pseudomonas* sp. N31 was isolated from soil and this strain will degrade, partially, 2NP and 4C2NP in the presence of succinate, forming nitrite, catechol and 4-chlorocatechol respectively; the latter two compounds were not further degraded.

Pseudomonas sp. N31 resembles the *P. putida* strain B2 isolated by Zeyer et al. (1984) as both organisms express a nitrophenol oxygenase which is active against 2NP and 4C2NP. However, the isolate of Zeyer et al. has catechol 1,2-dioxygenase and *cis,cis*-muconic acid cycloisomerase activity and therefore can degrade totally 2NP, whereas strain N31, lacking these activities, can use 2NP as a nitrogen source only. Both organisms exhibit a high nitrophenol oxygenase activity against 4C2NP, but even strain B2 does not convert further the 4-chlorocatechol (Zeyer et al. 1986).

The transconjugant strains reported here can utilize 4C2NP as sole source of carbon and nitrogen. The nitrophenol oxygenase of the recipient strain N31 oxidizes 4C2NP to form 4-chlorocatechol which is then mineralized by the halocatechol (pathway) enzymes of the donor strains *Pseudomonas* sp. B13 or *Alcaligenes eutrophus* JMP134.

A preliminary report of the construction of strain N31-1 has been made (Cost-Workshop, Dübendorf, Switzerland, Dec.1986); to ensure that the transfer of genes was from B13 to N31 as indicated by the nutritional properties of the transconjugant strains, we repeated the conjugation experiment with the antibiotic resistant mutants of the parent strains, designated N31-53 and B13-50 respectively. Similarly with the transconjugants of strains JMP134/JMP134-1 and N31, gene transfer was to N31. Apart from differences in the stability of the 4C2NP phenotype, the transconjugant strains of B13 and JMP134 with N31 behave similarly. The reason for the instability of the phenotype has not been investigated, but it is of interest that Friedrich et al. (1983) also noted that the stability of pJP4 transconjugants varied with the host harbouring the plasmid. The transconjugants grew at approximately the same rate on 4C2NP and all grew more slowly on 3CB than do the donor strains. This may be due to the reduced levels of some of the halocatechol degrading enzymes in the hybrid organisms (Table 3). Similarly, the low level of catechol 1,2-dioxygenase may account for the apparent accumulation of catechol during growth of the transconjugants on benzoate.

The decision to also use JMP134-1 as a donor strain of genes encoding haloaromatic degrading enzymes was made as it was uncertain whether these enzymes could be induced in JMP134 by 4CC or a later metabolite and as expression of those genes in JMP134-1 is constitutive (Pieper et al. unpublished work; Pieper 1986).

No significant difference, however, was found between the transconjugants formed when either JMP134 or JMP134-1 was used as donor (of genes encoding halocatechol degradation). As the halocatechol enzymes are also expressed in the JMP134-type transconjugants, our results clearly show that either 4CC or one of its metabolites must be the inducer of catechol 1,2-dioxygenase. That transconjugant strains obtained from matings with JMP134 or JMP134-1 did not grow on 24D was unexpected as the halocatechol degrading enzymes were expressed at a level sufficient to allow growth on 3CB. As shown by the experiments using 24D and 3CB as inducers, neither 24D monooxygenase nor 2,4-dichlorophenol (2,4DCP) hydroxylase were synthesized in strains N31-57 to 60.

Transfer of the plasmids form each of these four independently isolated transconjugants to strains of *A. eutrophus* JMP134 cured of pJP4, resulted in strains which were $24D^-$, $3CB^+$ and Hg^r and therefore the lack of expression of the above two enzymes in N31-57 to 60 is unlikely to be due to the N31-background. Therefore pJP4 must have undergone a mutation, either during conjugation or selection on 4C2NP, which has affected the synthesis of both enzymes. Recently Don et al. (1985) and Streber et al. (1987) have described six classes of transposon insertion mutants, which are defective in the synthesis of enzymes of the 24D pathway. Three of these classes, A, B and F have the same phenotype as the transconjugants isolated in this study, but the transconjugant strains differ biochemically from the transposon mutants as both 24D monooxygenase and 24DCP hydroxylase are non-inducible. Physical analysis of the plasmids in N31-57 to 60 will be necessary to determine the site(s) of any change in pJP4 responsible for the phenotype of the transconjugants.

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