Molecular cloning and expression of a novel catechol 2,3-dioxygenase gene from the benzoate *meta*-cleavage pathway in *Azotobacter vinelandii*

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Azotobacter vinelandii strain 206 degrades benzoate via the *meta*-cleavage pathway. In a genomic library derived from this organism a clone was obtained which carried and expressed the gene for the third enzyme in this pathway, catechol 2,3-dioxygenase (EC 1.13.11.2), on a 5.9 kb *Sal*I restriction fragment. The structural gene was more precisely mapped on an internal 1.6 kb *Eco*RI fragment which, after insertion into expression vectors, directed the synthesis of a 33 kDa polypeptide. The gene showed very little or no homology with isofunctional genes derived from *Pseudomonas*. Comprehensive substrate specificity analysis showed significant differences between the specific activities obtained from the cloned gene product and extracts derived from *Azotobacter* itself.

Introduction

Azotobacter is well-known for its efficient nitrogen fixation under obligatory aerobic conditions and has therefore been the target of extensive studies on its nitrogen fixation (*nif*) genes (Bishop *et al.*, 1986; Jacobsen *et al.*, 1986; Robson, 1986). Independent investigations into the architecture of the *Azotobacter* genome have revealed the presence of multiple genome copies for both *Azotobacter vinelandii* (Sadoff *et al.*, 1979) and *Azotobacter chroococcum* (Robson *et al.*, 1984), with a copy number between 20 and 40 per cell. This high degree of polyploidy is paralleled by the approximately 10-fold greater volume of the vegetative cells of *Azotobacter* when compared with those of *E. coli*. The chemistry and enzymology of aromatic catabolism have mainly been investigated in soil micro-organisms of the genus *Pseudomonas* (Dagley, 1986); these studies have been complemented by investigations into the molecular genetics of these catabolic traits, particularly where the genes were found to be plasmid-borne (Harayama & Don, 1985). Little information, however, is available about the catabolic activities of *Azotobacter*; the presence of a *meta*-cleavage pathway for the degradation of benzoate has been described in *A. vinelandii* strain 206 (Sala-Trepat & Evans, 1971) but there have been no

studies on the genetics of this phenotype. In this report the cloning and molecular analysis of the catechol 2,3-dioxygenase (C23DO) gene of the *meta*-cleavage pathway from this strain is described and results are presented demonstrating a low degree of homology with isofunctional genes and gene products derived from *Pseudomonas*.

Methods

Bacterial strains and plasmids. A list of strains used or constructed in this study is presented in Table 1.

Media and culture conditions. *A. vinelandii* strain 206 was grown on solid and liquid nitrogen-free minimal medium (Hardisson *et al.*, 1969) with sodium benzoate (15 mM) or sodium acetate (10 mM) as carbon sources. *E. coli* strains were cultivated in Luria broth supplemented with the appropriate antibiotic at the following concentrations ($\mu\text{g ml}^{-1}$): ampicillin, 25; tetracycline, 8; chloramphenicol, 30; kanamycin, 25.

Recombinant strains carrying expression vectors were grown at the appropriate temperature to an OD₆₀₀ of 0.55. Induction was brought about by the addition of IPTG (isopropyl β -D-thiogalactoside) (2 mM final concentration with pMMB22) or by a shift in temperature to 42 °C for 2 h with pPLGN1.

DNA extraction and purification. DNA was isolated from *Azotobacter* in a scaled-down modification of the procedure described by Sadoff *et al.* (1979). A 200 ml culture was grown up to an OD₅₇₈ of 1.0. Cells were spun down and resuspended in 1.6 ml 10 mM-Tris/HCl, 100 mM-NaCl, pH 8.1; 0.4 ml 120 mM-Tris/HCl, 50 mM-EDTA, 0.4 mg lysozyme ml⁻¹, pH 8.1, was added followed by 2.05 ml 1% (v/v) Triton X-100, 2 M-NaCl, 10 mM-EDTA, pH 8.1. The suspension was incubated for 10 min at 37 °C during which time cell lysis occurred. Proteins were removed by addition of 0.4 ml 5 M-sodium perchlorate and 4.5 ml

Abbreviations: C23DO, catechol 2,3-dioxygenase; DCCD, dihydroxycyclohexadiene carboxylate dehydrogenase; HMSD, 2-hydroxymuconic semialdehyde dehydrogenase; IPTG, isopropyl β -D-thiogalactoside.

Table 1. *Bacterial strains and plasmids*

Strain	Plasmid	Notes and reference
<i>Azotobacter vinelandii</i> strain 206		Sala-Trepat & Evans (1971)
<i>Alcaligenes eutrophus</i> mutant B9		Reiner & Hegeman (1971)
<i>E. coli</i> ED8654		<i>metB SupE SupF hsdR hsdM</i> (Borck <i>et al.</i> , 1976)
<i>E. coli</i> JM103		$\Delta(lac-pro) thi strA supE endA sbcB hsdR F' traD36 proAB lacI^q z$ M15 (Messing <i>et al.</i> , 1981)
<i>E. coli</i> C600	pBR322	Bolivar <i>et al.</i> (1977)
	pBR325	Bolivar (1978)
	pKT230	Bagdasarian <i>et al.</i> (1981)
	pMMB22	Bagdasarian <i>et al.</i> (1983)
	pPLGN1	Leeman <i>et al.</i> (1987)
	pEKA3	5.9 kb <i>SalI</i> fragment from <i>A. vinelandii</i> cloned in pBR322
	pEKA33	Reverse orientation of pEKA3 in pBR322
	pEKA41	1.6 kb internal <i>EcoRI</i> subfragment of pEKA3 inserted in pBR325
	pEKA43	1.5 kb internal <i>EcoRI</i> subfragment of pEKA3 inserted in pBR325
	pEKA45	2.1 kb internal <i>EcoRI</i> subfragment of pEKA3 inserted in pBR325
	pEKA3ptac	1.6 kb <i>EcoRI</i> subfragment of pEKA3 inserted into pMMB22
	pEKA3pL	Same fragment inserted into pPLGN1
	pEKP171ptac	2.1 kb <i>EcoRI</i> subfragment of pWW15-3171 carrying the C23DOII gene of <i>P. putida</i> MT15 (Keil <i>et al.</i> (1985a) inserted into pMMB22
	pEKP171pL	Same fragment inserted into pPLGN1

chloroform/isoamyl alcohol (97:3, v/v) and subsequent incubation at 25 °C for 15 min. The aqueous phase was separated by centrifugation and extracted twice more under the same conditions. DNA was precipitated at -20 °C by addition of 2 vols 95% (v/v) ethanol. The DNA pellet was redissolved in 2 ml TE-buffer (10 mM-Tris/HCl, 1 mM-EDTA pH 8.0) and treated with RNAase (50 µg ml⁻¹ final concentration) for 1 h at 37 °C and Pronase (100 µg ml⁻¹ final concentration) for 2 h at 37 °C. The DNA was extracted twice with phenol and the aqueous phase precipitated with 2 vols ethanol: the DNA pellet was finally dissolved in 200 µl TE and stored at 4 °C; 10 µg of the DNA was used for subsequent cloning experiments. Vector and cloned DNA were purified by CsCl/ethidium bromide equilibrium centrifugation whereas for small scale preparations of recombinant DNA the method of Holmes & Quigley was used (1981).

DNA cloning and manipulation. Restriction endonuclease cleavage and DNA ligations were done according to the instructions provided by the manufacturer; *E. coli* strains ED8654 and JM103 were transformed by standard procedures (Cohen *et al.*, 1972). Colonies carrying recombinant plasmids expressing C23DO activity were identified by the catechol spray test (Worsey *et al.*, 1978).

DNA-DNA hybridization. DNA restriction fragments were separated by agarose gel electrophoresis and transferred to Gene Screen Plus™ (Du Pont) by the Southern blotting technique. The inserted fragment was separated from the vector by agarose gel electrophoresis and purified by electroelution (Maniatis *et al.*, 1982). DNA was radiolabelled by nick-translation using [α -³²P]dGTP and hybridized as described previously (Keil & Williams, 1985) in 50% (v/v) formamide at 42 °C.

Enzyme analysis. Crude extracts of *A. vinelandii* and *E. coli* were prepared as described previously (Worsey & Williams, 1975). The enzyme assay procedures for C23DO and HMSD (hydroxymuconic semialdehyde dehydrogenase) were taken from Sala-Trepat & Evans (1971). DCCD (dihydroxycyclohexadiene carboxylate dehydrogenase) was assayed according to Reiner (1972). The substrate was prepared via biotransformation of benzoate using *Alcaligenes eutrophus* mutant B9 (Reiner & Hegeman, 1971).

PAGE. Denaturing 12% (w/v) acrylamide/bisacrylamide (30:0.5) gels were used to resolve total cell protein as described by Laemmli (1970).

Results

Cloning and physical mapping of C23DO genes from Azotobacter

Cleavage of *A. vinelandii* genomic DNA with *Bam*HI and *Hind*III was found to be limited, leaving most of the DNA unrestricted in the high-molecular-mass size range. Restriction with *Eco*RI, *Sal*I and *Xho*I, however, gave rise to a wide size range of fragments; hence the latter two enzymes were chosen for the construction of a genomic library from *A. vinelandii* in the two vectors pBR322 (Bolivar *et al.*, 1977) and pKT230 (Bagdasarian *et al.*, 1981). In a typical cloning experiment about 10⁴ transformants were obtained and subjected to the catechol spray test which allowed rapid identification of recombinant *E. coli* clones expressing the C23DO gene. From the *Sal*I cloning experiment one clone was obtained carrying the C23DO gene; none was detected in the *Xho*I cloning procedure. Analysis of the recombinant plasmid DNA in the C23DO-expressing clone revealed the presence of a 5.9 kb *Sal*I insert in plasmid pEKA3. In order to physically locate the structural gene more precisely on the cloned fragment subcloning experiments were done. Plasmid pEKA3 was restricted with *Eco*RI and the three internal fragments were inserted into

Fig. 1. Restriction map of the *SalI* fragment cloned from *A. vinelandii*. The black bar represents the location of the C23DO gene. The arrows indicate the direction of transcription from the promoter of the tetracycline-resistance gene on pBR322 (P_{tet}). The various subclones generated are shown below the map. The abbreviations for restriction enzyme sites are as follows: Av, *AvaI*; Bg, *BglII*; Bs, *BstEII*; E, *EcoRI*; Hc, *HincII*; P, *PstI*; S, *SalI*; Sm, *SmaI*; St, *StuI*. There are no sites for *AsnI*, *BamHI*, *HindIII*, *HpaI*, *KpnI*, *SacI*, *SphI*, *XbaI* and *XhoI*.

vector pBR325 generating the recombinant plasmids pEKA41, 43 and 45 (Fig. 1); the *SalI* insert was also reoriented in respect of the vector promoter to give pEKA33. The 1.6 kb *EcoRI* fragment was inserted into the expression vectors pMMB22 and pPLGN1 to generate pEKA3ptac and pEKA3pL respectively.

Enzyme activities of C23DO gene products

All enzyme studies on cloned genes were done in *E. coli*. The specific activity in strains carrying pEKA3 was

comparatively low (Table 2) although the gene was inserted such that transcription could be initiated from the constitutive tetracycline promoter of pBR322. No C23DO activity could be detected in strains containing pEKA33, with the 5.9 kb *SalI* insert in the opposite orientation, suggesting that transcription runs from left-to-right as shown in Fig. 1 and that there are no promoter sequences functional in *E. coli* upstream of the C23DO gene on pEKA3. By enzyme analysis of additional subclones of pEKA3 such as pEKA41 and pEKA3ptac the gene was mapped to the right-hand end of the cloned *SalI* fragment (Table 2 and Fig. 1). In *A. vinelandii* strain 206, C23DO is one of a set of enzymes the expression of which is induced in the presence of the growth substrate benzoate (Table 2; Sala-Trepat & Evans, 1971). Therefore cells containing pEKA3 were analysed for the activities of the enzymes which are adjacent in the catabolic pathway. Neither benzoate dioxygenase and DCCD, the first two enzymes of the pathway, nor HMSD, the enzyme acting on the product of C23DO activity, were detected; neither was there any significant increase in C23DO expression measured in *E. coli* upon addition of benzoate to the growth medium. By comparison, in *A. vinelandii* strain 206 all three enzyme activities were well-expressed and induced about 500-fold in the presence of benzoate (Table 2).

Analysis of substrate specificity

The specific activities of the C23DO encoded on pEKA3 and its derivatives towards catechol and the two

Table 2. Specific activities of catabolic enzymes involved in the degradation of benzoate

A. vinelandii strain 206 was cultivated either on sodium acetate or sodium benzoate. *E. coli* ED8654 carrying various recombinant plasmids was grown on Luria Broth. Enzyme activities are expressed as milliunits of activity (mg protein)⁻¹. Values in parentheses are the ratios of activity towards 3-methyl- and 4-methylcatechol with respect to the activity towards catechol. ND, Not determined.

Strain/plasmid	C23DO			DCCD	HMSD
	Catechol	3-Methylcatechol	4-Methylcatechol		
<i>A. vinelandii</i> 206 (acetate-grown)	2	< 1	< 1	< 1	1
<i>A. vinelandii</i> 206 (benzoate grown)	980	44 (0.04)	80 (0.08)	350	790
<i>E. coli</i> /pEKA3	7	1 (0.14)	0.4 (0.06)	< 1	< 1
<i>E. coli</i> /pEKA33	< 1	< 1	< 1	ND	ND
<i>E. coli</i> /pEKA41	168	27 (0.16)	9.5 (0.06)	ND	ND
<i>E. coli</i> /pEKA3ptac	450	45 (0.10)	16 (0.04)	ND	ND
<i>E. coli</i> /pEKA3ptac (+ IPTG)	4000	480 (0.12)	250 (0.06)	ND	ND
<i>E. coli</i> /pEKP171ptac	180	300 (1.66)	50 (0.27)	ND	ND
<i>E. coli</i> /pEKP171ptac (+ IPTG)	5500	9800 (1.77)	2600 (0.46)	ND	ND

Fig. 2. Hybridization pattern of genomic DNA from *A. vinelandii* and various recombinant plasmids carrying catechol-2,3-dioxygenase genes. (a) Ethidium-bromide-stained agarose gel; (b) autoradiogram of the same gel after hybridization to radiolabelled pEKA3 DNA. Lane 1, *A. vinelandii* genomic DNA restricted with *Eco*RI; lane 2, *A. vinelandii* DNA restricted with *Sal*I; lane 3, pEKA3 restricted with *Sal*I; lane 4, pWW15-3171 restricted with *Eco*RI; lane 5, *Xho*I fragment XI from pWW0 inserted in pKT230, restricted with *Xho*I. The absence of any hybridization signal in lane 4 and 5 suggests little or no similarity of the C23DO gene from *Azotobacter* with isofunctional catabolic genes from *Pseudomonas*.

methyated derivatives 3-methyl- and 4-methylcatechol were determined and compared with the specific activities measured in crude extracts prepared from *A. vinelandii* (Table 2). Cell extracts derived from pEKA3 all displayed similar specificity towards catechol, but showed very low activity towards 3-methylcatechol (ratio 0.12–0.16) and an even lower activity towards 4-methylcatechol (ratio 0.06). Crude extracts obtained from *A. vinelandii* showed a significantly different substrate specificity pattern in respect of 3-methylcatechol: the ratio of specific activities (3-methylcatechol with respect to catechol) was much lower (0.04) when compared with the equivalent ratios obtained for the cloned C23DO gene products on pEKA3. On the other hand, 4-methylcatechol was only a slightly better substrate with *Azotobacter* crude extracts (ratio 0.08) than with the pEKA3-derived extracts (Table 2). The relative substrate specificity ratios of the C23DOII gene derived from *P. putida* MT15 and inserted in pMMB22 were significantly higher when compared with the *Azotobacter* data (Table 2) and in agreement with

previous results obtained using different host–vector systems (Keil *et al.*, 1985a), suggesting a less-restricted substrate specificity for this enzyme.

DNA–DNA hybridization of the *Azotobacter* C23DO gene with *Pseudomonas* C23DO genes

In order to assess the degree of homology between the C23DO gene cloned from *Azotobacter* and genes coding for isofunctional enzymes previously cloned from *Pseudomonas* TOL plasmids (Inouye *et al.*, 1981; Keil *et al.*, 1985a) the 5.9 kb *Sal*I insert of pEKA3 was radiolabelled and used as a probe in DNA–DNA hybridization experiments (Fig. 2). In the *Sal*I-restricted genomic DNA from *A. vinelandii* a 5.9 kb *Sal*I restriction fragment did hybridize together with a few bands of > 9 kb (lane 2). A 1.6 kb *Eco*RI band hybridized in *Eco*RI-digested *Azotobacter* DNA corresponding to the internal *Eco*RI fragment of pEKA3. The larger bands hybridizing in the *Sal*I restricted genomic DNA (lane 2) may suggest the presence of additional copies of the

Fig. 3. SDS-PAGE of total proteins of recombinant *E. coli* strains carrying cloned C23DO genes that had (lanes 3, 5, 7, 9 and 11) or had not (lanes 2, 4, 6, 8 and 10) been induced. The samples were: pEKA3ptac (lanes 2 and 3); pEKP171ptac (lanes 4 and 5); pEKA3pL (lanes 6 and 7); pEKP171pL (lanes 8 and 9); *E. coli* ED8654 (lanes 10 and 11). Lanes 1 and 12 contained standard molecular mass markers (from top to bottom: 97.4 kDa, 66.2 kDa, 42.7 kDa, 31 kDa, 21.5 kDa and 14.4 kDa).

CDO gene located on conserved 1.6 kb internal *EcoRI* fragments; this is supported by the relatively strong hybridization signal shown by this restriction fragment in lane 1. No clear positive signal was detected in the two lanes with the recombinant plasmids carrying C23DO genes derived from the *Pseudomonas* TOL plasmids pWW0 (*xylE* gene) or pWW15 (C23DOI gene), respectively.

Expression of C23DO genes cloned from Pseudomonas and Azotobacter

The 2.1 kb *EcoRI* fragment from the recombinant plasmid pWW15-3171, known to carry the structural gene of the C23DO originally cloned from *P. putida* MT15 (Keil *et al.*, 1985a), was inserted into the expression vectors pMMB22 and pPLGN1 to give rise to pEKP171ptac and pEKP171pL, respectively. Together with the corresponding clones pEKA3ptac and pEKA3pL carrying the C23DO gene from *A. vinelandii*, the four recombinant plasmids were transformed in *E. coli* ED8654; the protein profiles of cells that had or had

not been induced were subsequently analysed by SDS-PAGE. A protein of approximate molecular mass 33 kDa was detected in extracts of all induced cells (Fig. 3). The two proteins hence appeared to be slightly smaller than the C23DO purified from *P. putida* mt-2 with an M_r of 35000 (Nakai *et al.*, 1983).

Discussion

This paper describes a new type of C23DO gene present in *Azotobacter* and unrelated to isofunctional genes isolated mainly from *Pseudomonas*. Previous investigations on C23DO genes from the latter genus have shown that one type appears to be widespread among different natural isolates of *Pseudomonas*. This gene was originally cloned from TOL plasmid pWW0 and called *xylE* (Inouye *et al.*, 1981) and was subsequently found in similar form in other non-related TOL plasmids (Keil *et al.*, 1985a, b; Chatfield & Williams, 1986; Shaw & Williams, 1988) or in plasmids coding for the catabolism of naphthalene (Lehrbach *et al.*, 1983; Assinder & Williams, 1988). Most of these comparative studies have been done by DNA-DNA hybridization but comparative nucleotide sequence analysis showed a 96% similarity between *xylE* of pWW0 and the C23DOI gene of pWW15 (H. Keil & P. A. Williams, unpublished results) and 80% similarity between *xylE* and *nahH*, a gene coding for an isofunctional enzyme in the naphthalene catabolic pathway on plasmid NAH7 (Ghosal *et al.*, 1987; Harayama *et al.*, 1987). A completely different C23DO gene has been identified on the non-transmissible TOL plasmid pWW15 sharing very little or no identity with the archetypal *xylE* gene (Keil *et al.*, 1985a). A third and different type of C23DO gene has now been cloned from *Azotobacter*. These findings could imply that the structural gene for C23DO has evolved independently several times with the *xylE* type being the most ubiquitous version because of its location on self-transmissible broad-host-range plasmids such as pWW0 or NAH7 (Benson & Shapiro, 1978; Dunn & Gunsalus, 1973) and/or its presence on the catabolic transposon Tn4561 (Tsuda & Iino, 1987). However, a common ancestor for these non-homologous genes in the distant past cannot be ruled out until information about their nucleotide sequence is available.

The absence of any activity of other enzymes from the catabolic pathway, in particular within the 4 kb region upstream of the C23DO gene in pEKA3, can be explained in two ways. Either the structural genes of the pathway are widely dispersed on the *Azotobacter* genome or, alternatively, the genes are located in two distinct clusters analogous to the situation in *Acinetobacter* where the first three genes *benA, B, C* coding for the enzymes converting benzoate to catechol are physically separated

on the genome from the genes coding for the ortho-cleavage pathway *catA,B,C,D*... converting catechol to 3-oxoadipate (Shanley *et al.*, 1986; Neidle *et al.*, 1987). In any case the absence of a tightly spaced catabolic gene cluster, characteristic of plasmid-encoded functions, suggests a chromosomal location for the catabolic genes in *Azotobacter*, confirmed by the absence of any plasmid DNA in strain 206 (unpublished data).

The stringent specificity of the cloned C23DO gene product for unsubstituted catechol only is not surprising since *A. vinelandii* shows poor growth on substituted benzoates such as *m*-toluate. By contrast, *P. putida* grows readily on *m*-toluate and its isofunctional enzymes display a nearly equivalent or even higher affinity towards 3-methylcatechol than towards catechol (Keil *et al.*, 1985a; Table 2). The fact that crude extracts obtained from *A. vinelandii* show a significantly different substrate specificity compared with the cloned gene product raises the possibility of the presence of additional, as yet unidentified, C23DO genes in this organism. Three homologous copies of the *nifH* gene coding for dinitrogen reductase have been identified by DNA-DNA hybridization on the genome of *Azotobacter vinelandii* (Jacobsen *et al.*, 1986) and from the same organism three dinitrogenases with different cofactor requirements (molybdenum, vanadium and iron) and expressed under different growth conditions have been isolated (Bishop *et al.*, 1980; Chishell *et al.*, 1988). The question as to the presence of multiple copies of catabolic genes in *Azobacter* is currently being addressed by constructing and screening a random genomic library in cosmid vectors.

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