Complementation of *Xanthobacter* Py2 mutants defective in epoxyalkane degradation, and expression and nucleotide sequence of the complementing DNA fragment

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Three *Xanthobacter* Py2 mutants (M3, M8 and M10) lacking epoxyalkane-degrading activity were isolated and characterized. All mutants were able to grow on acetone, the degradation product of 1,2-epoxypropane conversions. Furthermore, they contained the unidentified 'low molecular mass fraction' (LMF) necessary for epoxyalkane-degrading activity. Three cosmids from a gene bank complemented the mutation in M10 and M8 but not in mutant M3. Epoxyalkane-degrading activity in crude extracts of 1,2-epoxypropane-grown complemented mutants was similar to the wild-type activity. Surprisingly, M10 transformed with complementing cosmid pEP9 showed a constitutively expressed epoxyalkane-degrading activity, which was not observed in the wild-type strain. The cosmid pEP9 was conjugated into *Xanthobacter autotrophicus* GJ10, which is not able to degrade 1,2-epoxypropane. In crude extracts of *X. autotrophicus* GJ10(pEP9), epoxyalkane-degrading activity was demonstrated, but only after the addition of the LMF from *Xanthobacter* Py2. Hybridization experiments demonstrated an overlap on complementing cosmids pEP1, pEP3 and pEP9. Subcloning revealed a 4.8 kb *EcoRI*–*HindIII* fragment to be necessary for complementing the mutant M10. In the sequence of this fragment four different ORFs were found.

Keywords: *Xanthobacter* Py2, epoxyalkane degradation, complementation

INTRODUCTION

Several methods have been described to produce optically pure epoxides by biological methods (Weijers *et al.*, 1988b; Leak *et al.*, 1992; De Bont, 1993). One such method is the enantioselective degradation of racemic epoxides; however, such a process yields a maximum of 50% product. *Xanthobacter* Py2 was isolated on propene as sole carbon and energy source (Van Ginkel & De Bont, 1986). This strain is able to degrade the (2S)-forms of *trans*-2,3-epoxybutane and 2,3-epoxypentane, resulting in optically pure (2R)-2,3-epoxyalkanes; the C₃–C₆ 1,2-epoxyalkanes are degraded completely (Weijers *et al.*, 1988a).

Abbreviation: LMF, low molecular mass fraction.

The EMBL accession number for the sequence reported in this paper is X79863.

Ketones have been identified as degradation products of epoxyalkane metabolism in crude extracts of propene-grown cells (Fig. 1). It has been demonstrated that both NAD and an unidentified 'low molecular mass fraction' (LMF) are involved in the reaction. In a reaction mechanism proposed by Weijers *et al.* (1994) it was suggested that the LMF is involved in the reduction of the epoxyalkanes to secondary alcohols since the LMF can be replaced by reducing compounds like DTT and other dithiol compounds. The presence of NAD is necessary for the oxidation of the alcohols to the corresponding ketones.

Attempts to purify the epoxyalkane-degrading enzyme have not been successful. Consequently, we have now investigated the genetics of epoxyalkane degradation in *Xanthobacter* Py2. Eventually, we hope to be able to obtain the enzyme to study further its reaction mechanism.

In this study we report the selection and characterization

Fig. 1. Proposed degradation pathway of epoxyalkanes by *Xanthobacter* Py2. X-(SH)₂ represents the LMF necessary for epoxyalkane degradation (Weijers *et al.*, 1994).

of mutants devoid of epoxyalkane-degrading activity, the complementation of these mutants and the expression of one of the complementing cosmids in *Xanthobacter autotrophicus* GJ10. In addition, a 4.8 kb fragment complementing the M10 mutation was sequenced and analysed.

METHODS

General methods. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* cells were grown on LB medium (Sambrook *et al.*, 1989). *Xanthobacter* strains were grown at 30 °C on yeast extract-glucose medium (10 g l⁻¹ each) or in sealed flasks with mineral medium (Wiegant & De Bont, 1980) to which 5 mM volatile compound was added to the water phase or 5% (v/v) propene added to the gas phase. For induction of epoxyalkane-degrading activity, cells were grown in yeast extract-glucose medium (750 ml) in sealed 5 l flasks to which 1% propene was added to the gas phase as inducing agent. Cells

cultivated on plates were placed in a desiccator when a volatile compound was used as a substrate; for each plate (20 ml) 0.1 mmol of the substrate was added to give a final concentration of 5 mM. The antibiotics used for the selection of plasmids were tetracycline (12.5 mg ml⁻¹), ampicillin and kanamycin (both 50 mg ml⁻¹). Preparation of crude extracts, and LMF, and the activity assay for epoxyalkane degradation in which the initial degradation rate of 1,2-epoxypropene in extracts is followed using head-space GC analysis, have been described by Weijers *et al.* (1994).

Mutant isolation. Exponentially growing cultures (5 ml) of *Xanthobacter* Py2 grown on propene were UV-irradiated until less than 1% of the cells survived. The cells were placed in the dark in 20 ml liquid mineral medium with 5 mM acetone until growth was visible. The cells were harvested by centrifugation at 15000 g for 5 min, washed and resuspended in the same volume of mineral medium containing 1,2-epoxypropene. Glycine (2%, w/v) was added after 8 h incubation to kill growing cells. After overnight incubation in the glycine-containing medium, the cells were harvested and washed in sterile demineralized water. Recovery, starvation and killing of the cells was repeated once more before spreading them on yeast extract-glucose plates.

Gene bank preparation. Total DNA was prepared from propene-grown wild-type *Xanthobacter* Py2 cells. Cells from 750 ml cultures were harvested, resuspended in 25 ml TEG (10 mM Tris, 1 mM EDTA, 50 mM glucose, pH 8.0) and lysed by the addition of SDS (2%, w/v, final concentration). Proteins were removed by extraction with phenol/chloroform, and DNA was precipitated with 2-propanol. Purified DNA was dissolved in TE buffer, partially digested with *Sau3A* and dephosphorylated. The two pLAFR5 cosmid arms were prepared by digestion with *ScaI* and *BamHI* (Keen *et al.*, 1988). Ligated DNA was packaged using the Packagene *in vitro* packaging system supplied by Promega and used to transduce *E. coli* LE392; the transduced cells were spread on plates (100–200 colonies per plate). After overnight growth cells were resuspended and pooled in LB supplemented with 30% (v/v) glycerol and stored at –80 °C.

Triparental mating. This was carried out essentially as described by Janssen *et al.* (1989) but instead of NB agar, yeast extract-

Table 1. Bacterial strains and cosmids used

Organism or cosmid	Relevant properties	Reference
<i>E. coli</i>		
LE392	RecA ⁻	Sambrook <i>et al.</i> (1989)
TG1	RecA ⁻	Sambrook <i>et al.</i> (1989)
HB101	RecA ⁻	Boyer & Roulland-Dussoix (1969)
<i>Xanthobacter</i>		
Py2	Epoxyalkane-using wild-type	Van Ginkel & De Bont (1986)
Py2 M3	Defective in epoxyalkane degradation	This study
Py2 M8	Defective in epoxyalkane degradation	This study
Py2 M10	Defective in epoxyalkane degradation	This study
<i>X. autotrophicus</i> GJ10	Growth on acetone, not on epoxyalkanes	Janssen <i>et al.</i> (1985)
Cosmids		
pLAFR5	Tc ^r RK2 replicon <i>mob</i> ⁺	Keen <i>et al.</i> (1988)
pEP1, -3, -9	pLAFR5 containing <i>Xanthobacter</i> Py2 DNA	This study
pRK2013	<i>tra</i> (RK2) ColE1 replicon Km ^r	Figurski & Helinski (1979)
pT7/T3-19	Ap ^r pUC19 derivative	Life Technologies

glucose agar was used. The conjugated recipient strains were selected on tetracycline-containing mineral medium agar plates with either 1,2-epoxypropane or 2-propanol as substrate.

DNA manipulations, Southern transfer, hybridization and nucleotide sequencing. Standard recombinant DNA techniques were performed as described by Sambrook *et al.* (1989). DNA was isolated from agarose gel using GeneClean II (Bio 101). For Southern transfer, digested cosmid DNA was separated on a 0.7% agarose gel, blotted onto a nylon membrane (Nytran NY 13N; Schleicher & Schuell) by capillary transfer and fixed on the membrane by UV-irradiation for 2 min. Probes were prepared using the DIG DNA labelling and detection kit (Boehringer). Hybridization and detection were performed using the same kit, except that the hybridization temperature was 71 °C. A 4.8 kb *EcoRI*–*HindIII* fragment was ligated into plasmid pT7/T3a-19. The insert was sequenced in both directions by Pharmacia Biotech Benelux using the primer walking method. Nucleotide sequencing was performed using the dideoxy chain-termination method (Sanger *et al.*, 1977) using 5'-FITC-labelled primers. Computer analysis of the sequence was accomplished with the Staden program.

RESULTS

Isolation of *Xanthobacter* Py2 mutants devoid of epoxyalkane-degrading activity

The complementation method used for the isolation of *Xanthobacter* Py2 DNA sequences involved in epoxyalkane degradation by *Xanthobacter* Py2 requires mutants defective in the protein responsible for epoxyalkane degradation. To ensure that only this protein is mutated and not enzymes further down the degradation pathway, these mutants should still be able to grow on acetone, the first detectable product of 1,2-epoxypropane degradation. These mutants should also contain the unidentified 'low molecular mass fraction' (LMF) required for epoxide degradation.

Mutants were obtained as described in Methods. Single colonies were streaked on mineral medium plates and incubated for 1 week in a desiccator containing 1,2-epoxypropane. Wild-type cells able to grow on 1,2-epoxypropane formed large slimy colonies while mutant cells formed very small colonies. Of 450 putative mutants tested, 23 did not grow on 1,2-epoxypropane. These small colonies were sub-cultured twice on mineral medium plates with 1,2-epoxypropane as substrate. This procedure resulted in 11 mutants unable to grow on 1,2-epoxypropane. These 11 mutants were tested for growth in liquid medium containing 1,2-epoxypropane; three mutants did not grow. These mutants, designated M3, M8 and M10, all grew on acetone with growth rates comparable to wild-type *Xanthobacter* Py2.

Characterization of mutants defective in epoxyalkane degradation

The three mutants devoid of epoxyalkane-degrading activity were tested for the presence of the LMF. In wild-type *Xanthobacter* Py2 no epoxyalkane-degrading activity was found when cells were grown on yeast extract-glucose medium; however, when 1% propene was added to the head-space during growth on yeast extract-glucose,

low activities were observed [approx. 2 nmol 1,2-epoxypropane degraded min⁻¹ (mg protein)⁻¹]. Crude extracts of mutant cells grown on yeast extract-glucose in the presence of 1% propene showed no epoxyalkane-degrading activity. These crude extracts were used for the preparation of the LMF and the protein fraction. By combining the protein fraction of the wild-type Py2 grown on 1,2-epoxypropane with the LMF of a mutant it is possible to detect whether the LMF is present in the mutant. The combination of the protein fraction of the wild-type Py2 with the LMF of the mutants resulted in epoxyalkane-degrading activity of between 4 and 6 nmol 1,2-epoxyalkane degraded min⁻¹ (mg protein)⁻¹. The specific activity of combined wild-type *Xanthobacter* Py2 LMF and protein fraction was 12 nmol 1,2-epoxyalkane degraded min⁻¹ (mg protein)⁻¹. No activity was detected when the LMF of the wild-type *Xanthobacter* Py2 was combined with the protein fraction of the mutant cells. These results show that the LMF is still present in the three mutants and that the protein fractions of the mutants are inactive.

Complementation of *Xanthobacter* Py2 mutants

The broad-host-range cosmid pLAFR5 was used as a cloning vector for *Xanthobacter* Py2 because of its ability to replicate in *Xanthobacter* species (Janssen *et al.*, 1989). The vector contains a double *cos* site, allowing the insertion of relatively large DNA fragments.

Screening of the gene bank with an average insert size of 17 kb was done by individually transferring the recombinant cosmids from the transduced *E. coli* LE392 to the recipient mutant M10 using triparental mating as described in Methods. The conjugated recipients were selected for growth on mineral medium plates in the presence of tetracycline using 1,2-epoxypropane as substrate. Nine tetracycline-resistant colonies were able to grow on the substrate. To rule out reversion of the mutation and to eliminate unstable cosmids, the nine cosmids were isolated from these complemented M10 colonies, used to transform *E. coli* TG1 and again conjugated into M10. Five out of nine cosmids (pEP1, pEP3, pEP4, pEP5 and pEP9) restored the ability to grow on 1,2-epoxypropane both on plates and in liquid mineral medium.

Restriction enzyme analysis of the five different cosmids with *Bam*HI, *Xho*I, *Eco*RI and *Sal*I revealed that the restriction pattern of pEP3 was identical to pEP4; the same was observed for pEP5 and pEP9, leaving three different cosmids (pEP1, pEP3 and pEP9) complementing M10. The insert sizes of the three cosmids were 24, 26 and 22 kb for pEP1, pEP3 and pEP9, respectively.

The three cosmids were conjugated into the mutants M3 and M8. They complemented M8 as seen from good growth on plates in the presence of 1,2-epoxypropane. The M3 mutant, however, was not complemented by the cosmids. A subsequent conjugation of 2200 clones from the gene bank into M3 did not yield any cosmid restoring its ability to grow on 1,2-epoxypropane.

Fig. 2. Restriction map of the 22 kb insert of pEP9. The lines at the top represent the probes used for Southern hybridization with pEP1, pEP3 and pEP9 (Fig. 3). Restriction enzymes: B, *Bam*HI; E, *Eco*RI; X, *Xho*I; H, *Hind*III. The sites for restriction enzymes in italics belong to the multiple cloning site of the vector. (b) Deletion clones of pEP9. pEP9.1 is a *Bam*HI and pEP9.2 a *Eco*RI deletion clone.

Epoxyalkane-degrading activity in complemented mutant cells

Epoxyalkane-degrading activity was measured in crude extracts of mutants M8 and M10 grown under different conditions. The activity of cells grown on 1,2-epoxypropane was comparable to that of wild-type *Xanthobacter* Py2 [13 ± 2 nmol min⁻¹ (mg protein)⁻¹]. Surprisingly, the M8 and M10 mutants complemented with pEP9 showed a high epoxyalkane-degrading activity of 22 nmol min⁻¹ (mg protein)⁻¹ when grown on yeast extract-glucose medium in the absence of 1,2-epoxypropane, indicating constitutive expression. This was not observed in wild-type *Xanthobacter* Py2 cells nor in the mutants M8 and M10 complemented with pEP1 or pEP3.

Cofactor-dependent epoxyalkane-degrading activity in *X. autotrophicus* GJ10

The plasmid pEP9, which constitutively expressed epoxyalkane-degrading activity in M8 and M10, was conjugated into *X. autotrophicus* GJ10. This *Xanthobacter* strain is not able to grow on epoxyalkanes. Conjugants were selected on mineral medium plates containing tetracycline and 2-propanol. The conjugated *X. autotrophicus* GJ10 was not able to grow on 1,2-epoxypropane and no epoxyalkane-degrading activity was detected in crude extracts of cells grown on yeast extract-glucose. However, 1,2-epoxypropane was degraded by these extracts [2 nmol 1,2-epoxypropane degraded min⁻¹ (mg protein)⁻¹] if the LMF prepared from propene-grown *Xanthobacter* Py2 cells was added. No such activity was found when the LMF was added to crude extracts of wild-type *X. autotrophicus* GJ10.

Restriction map of pEP9 and hybridization studies with pEP1 and pEP3

Because pEP9 carries the information for epoxyalkane-degrading activity, as seen from *X. autotrophicus* GJ10 conjugated with pEP9, this cosmid was further charac-

Fig. 3. Southern hybridization studies of *Xho*I-digested pEP1 (lane 1), pEP3 (lane 3) and pEP9 (lane 9). (b) is the blot of digest (a) hybridized with probe 1 (Fig. 2). (c) and (d) are the digest and the blot hybridized with probe 2. A λ /HindIII marker was used.

terized. A restriction map of pEP9 was constructed based on migration patterns of digested pEP9 and on Southern hybridization of digests of pEP9 with several probes from the pEP9 insert (Fig. 2).

Southern hybridization of *Xho*I digests of pEP1, pEP3 and pEP9 are shown in Fig. 3. The blots were hybridized to two different probes of pEP9 (Fig. 2). Probe 1 is a 2.5 kb *Eco*RI-*Xho*I fragment (from 8 kb to 10.5 kb on the map) and probe 2 is a 1.4 kb *Xho*I fragment (from 19.6 kb to 21 kb on the map). Probe 1 hybridized only to a 5.2 kb *Xho*I fragment of pEP3, whereas probe 2 hybridized to both pEP1 and pEP3. Further restriction analyses with *Xho*I (Fig. 3), *Eco*RI, *Sal*I and *Bam*HI showed that pEP3 is homologous to pEP9 from approximately 5 kb to 22 kb on the pEP9 map. The cosmid pEP1 showed considerably less homology with both pEP3 and pEP9. Restriction homology with *Xho*I (Fig. 3) and *Sal*I was only found from the 18 kb point to approximately the 21.5 kb point of cosmid pEP9.

Subcloning of pEP9

The homology studies with pEP1 in particular indicated that the complementing area was present on the right-hand end of pEP9 (Fig. 3). Therefore, this part of pEP9 was used to construct subclones. Cosmid pEP9 was digested with *Bam*HI or *Eco*RI and religated to delete the internal fragments. The resulting cosmids were checked by restriction analysis and assayed for complementation of mutant M10. The *Bam*HI and the *Eco*RI deletion clones of pEP9, designated pEP9.1 and pEP9.2, respectively, had the ability to complement mutant M10 (Fig. 2). The nucleotide sequence of the complementing 4.8 kb *Eco*RI-*Hind*III fragment of pEP9.2 was determined.

Nucleotide sequence of the 4.8 kb complementing fragment of pEP9.2

The nucleotide sequence is presented in Fig. 4. The G + C content of the fragment was 65.4 mol%, which is lower than the 70 mol% found in *Xanthobacter* Py2 (Van Ginkel & De Bont, 1986). Four different ORFs were found, using the codon preference normally observed for *Xanthobacter* DNA. The four ORFs code for proteins of 41 690, 7388, 57315 and 26111 Da, respectively. In the opposite strand no ORFs of significant length were found.

A computer search of the Swiss-Prot protein bank did not reveal homology for the first two ORFs. But, interestingly, ORF3 showed significant homology with mercury(II) reductase (EC 1.16.1.1) from *Bacillus* strain RC607 (P16171), glutathione reductase (NADPH) (EC 1.6.4.2) from *Pseudomonas aeruginosa* (P23189) and dihydrolipoamide dehydrogenase (EC 1.8.1.4) from pig (P09623). For ORF4, homologies were found with 3-oxoacyl reductase (EC 1.1.1.35) from *Cuphea lanceolata* (P28643) and glucose 1-dehydrogenase (EC 1.1.1.47) from *Bacillus subtilis* (P12310).

DISCUSSION

Three major problems are encountered concerning the isolation of the gene(s) responsible for epoxyalkane

degradation in *Xanthobacter* Py2. The first is the absence of a purified enzyme (Weijers *et al.*, 1994). The second is the low specific activity of the enzyme: in crude extracts with 1,2-epoxypropane as substrate epoxyalkane-degrading activity was only 13 nmol min⁻¹ (mg protein)⁻¹. With *trans*-2,3-epoxybutane, which is degraded enantioselectively, this activity is even lower [± 1 nmol per min⁻¹ (mg protein)⁻¹]. Consequently, 1,2-epoxypropane was chosen as the model substrate to monitor epoxide-degrading activity. The third problem is the cofactor requirement in epoxyalkane degradation. Apart from NAD, another unidentified factor is necessary for activity. This cofactor requirement makes the isolation of the epoxyalkane-degrading protein or expression of the cloned genes in other strains more difficult. Recently it has been demonstrated that DTT can replace the unknown cofactor (Weijers *et al.*, 1994). However, in the present study the low molecular mass fraction (LMF) of crude cell extracts which contain the cofactor has been used rather than DTT to avoid any possible artefacts.

In view of the above difficulties, it was decided to isolate mutants lacking epoxyalkane-degrading activity which were subsequently used to isolate by complementation the gene(s) involved in epoxyalkane degradation. The procedure for creating and selecting mutant *Xanthobacter* strains was successful: the three mutants selected met all the requirements for use in complementation studies. They were unable to grow on 1,2-epoxypropane, but grew on acetone, the product of 1,2-epoxypropane degradation, and they contained the LMF.

Three different cosmids were found to restore the ability of mutant M10 to grow on 1,2-epoxypropane. These cosmids were also able to complement mutant M8. The epoxyalkane-degrading activities of the complemented mutants M8 and M10 were comparable to the wild-type *Xanthobacter* Py2. The finding that mutant M3 was not complemented by the cosmids suggests that it belongs to a complementation group different from M8 and M10. However, the inability to complement this mutant with the *Xanthobacter* Py2 gene bank shows that the M3 mutation is more complex.

Complementation of mutants lacking epoxyalkane-degrading activity does not itself necessarily imply that the structural genes for the degrading enzymes have been cloned. To determine whether this was the case cosmid pEP9 was conjugated into *X. autotrophicus* GJ10. Although *X. autotrophicus* GJ10(pEP9) did not grow on 1,2-epoxypropane, the protein required for epoxyalkane-degrading activity was present in extracts of cells grown on yeast extract-glucose. This was demonstrated by combining LMF obtained from wild-type *Xanthobacter* Py2 with the protein fraction of *X. autotrophicus* GJ10(pEP9), which resulted in 1,2-epoxypropane degradation and acetone formation.

The ORF(s) encoding the protein(s) involved in epoxyalkane degradation remain to be identified. The epoxide-degrading enzyme has been characterized only to a limited extent, but a reaction mechanism has been proposed (Weijers *et al.*, 1994). The mechanism suggested involves

Fig. 4. For legend see facing page.

the reduction of a disulfide bridge in the catalytic centre of the enzyme. Similarities were noted with vitamin K epoxide reductase and very recently the involvement of lipoamide reductase was implied in supplying reducing equivalents (Thyssen *et al.*, 1994). It is therefore very interesting that ORF3 shows significant homology with lipoamide reductase.

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