Degradation of trichloroethene by a linear-plasmid-encoded alkene monooxygenase in *Rhodococcus corallinus* (*Nocardia corallina*) B-276

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Rhodococcus corallinus (formerly *Nocardia corallina*) B-276, isolated with propene as sole carbon and energy source, is able to oxidize trichloroethene (TCE). Glucose- or propene-grown *R. corallinus* B-276 cells exhibited no difference in TCE degradation efficiency. TCE degradation was found to be growth-phase-dependent and maximum rates were monitored with stationary-phase cells. K_m and V_{max} values for TCE degradation of *R. corallinus* B-276 grown in nutrient broth medium in the presence of glucose were 187 μ M and 2.4 nmol min⁻¹ (mg protein)⁻¹, respectively. *Escherichia coli* recombinants harbouring and expressing the alkene monooxygenase genes of *R. corallinus* B-276 exhibited the ability to degrade TCE. This result provides clear evidence that the alkene monooxygenase of *R. corallinus* B-276 catalyses TCE oxidation. *R. corallinus* B-276 was shown to contain four linear plasmids, pNC10 (70 kb), pNC20 (85 kb), pNC30 (185 kb) and pNC40 (235 kb). The observation that pNC30-deficient strains had lost the ability to grow on propene suggested that the genes of the propene degradation pathway are encoded by the linear plasmid pNC30. Southern blot analysis with cloned alkene monooxygenase genes from *R. corallinus* B-276 revealed a positive hybridization signal with the linear plasmid pNC30. This result clearly shows that the alkene monooxygenase is encoded by the linear plasmid pNC30. Eleven short-chain-alkene-oxidizing strains were screened for the presence of linear plasmids. Among these, four propene-oxidizing *Rhodococcus* strains and one ethene-oxidizing *Mycobacterium* strain were found to contain linear megaplasmids. Southern blot analysis with the alkene monooxygenase revealed positive signals with linear plasmids of two propene-oxidizing *Rhodococcus ruber* strains. These results indicate that homologous alkene monooxygenases are encoded by linear plasmids in *R. ruber* strains.

Keywords: *Rhodococcus corallinus* (*Nocardia corallina*) B-276, alkene monooxygenase, trichloroethene cooxidation, linear plasmids

INTRODUCTION

Trichloroethene (TCE) is an extensively used industrial solvent and due to its broad application and high volatility it has become a major ground water con-

taminant. Since TCE is a potential health hazard, intensive efforts have been undertaken to analyse microbial TCE degradation and to develop appropriate bioremediation processes (Ensley, 1991). Several organisms have been shown to degrade TCE via non-specific catabolic oxygenases, such as methane monooxygenase of methanotrophs (Brusseau *et al.*, 1990; Fox *et al.*, 1990; Harker & Kim, 1990; Koh *et al.*, 1993; Oldenhuis *et al.*, 1989; Tsien *et al.*, 1989), ammonia

Abbreviations: CHEFE, contour-clamped homogeneous electric field electrophoresis; TCE, trichloroethene.

monooxygenase of *Nitrosomonas europaea* (Arciero *et al.*, 1989; Vannelli *et al.*, 1990), toluene 2-monooxygenase of *Burkholderia* (formerly *Pseudomonas*) *cepacia* G4 (Newman & Wackett, 1997; Shields *et al.*, 1989), toluene 3-monooxygenase of *Ralstonia* (formerly *Pseudomonas*) *pickettii* PKO1 (Kaphammer *et al.*, 1990), toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 (Winter *et al.*, 1989), toluene 2,3-dioxygenase of *Pseudomonas putida* F1 (Wackett & Gibson, 1988; Zylstra *et al.*, 1989), phenol hydroxylase of *Alcaligenes eutrophus* JMP134 (Kim *et al.*, 1996), alkene monooxygenase of *Xanthobacter* Py2 (Ensign *et al.*, 1992), isopropylbenzene dioxygenase of *Pseudomonas* sp. JR1 and *Rhodococcus erythropolis* BD2 (Dabrock *et al.*, 1992; Kessler *et al.*, 1996) and propane monooxygenase of *Mycobacterium vaccae* JOB5 (Wackett *et al.*, 1989).

Alkene monooxygenases catalyse the epoxidation of short-chain alkenes to their corresponding epoxides as the initial step in the utilization of aliphatic alkenes. The multicomponent alkene monooxygenase system of *Xanthobacter* Py2, which has been purified and characterized in some detail (Small & Ensign, 1997), has undergone intensive investigations with respect to degradation of chlorinated alkenes (Ensign *et al.*, 1992; Ensign, 1996).

Rhodococcus corallinus (formerly *Nocardia corallina*) B-276, which was isolated with propene as sole carbon and energy source, also initiates the oxidation of alkenes to the corresponding epoxides by an alkene monooxygenase (EC 1.14.13.-) (Furuhashi, 1992). The alkene monooxygenase of this strain, which has been purified and characterized, shows fundamental differences to the enzyme system of *Xanthobacter* Py2 (Miura & Dalton, 1995). The enzyme system of *R. corallinus* B-276 comprises three components: an epoxygenase, a NADH reductase and a coupling protein. The epoxygenase consists of two different subunits, a 53 and a 35 kDa subunit, and contains approximately 2 mol non-haem iron per mol protein. The reductase is an iron-sulfur flavoprotein of 40 kDa which is responsible for the transfer of reducing equivalents from NADH to the epoxygenase. The coupling protein is a single-subunit protein of 14 kDa not containing any prosthetic groups and appears to function as a regulator of activity (Miura & Dalton, 1995). Since the epoxygenase is considered to be the catalytic component of this enzyme system, its physico-chemical features have been closely investigated, leading to a proposal for the mechanism of stereospecific epoxidation by the alkene monooxygenase in *R. corallinus* B-276 (Gallagher *et al.*, 1997, 1998). Recently, Gallagher *et al.* (1997) showed that the alkene monooxygenase from *R. corallinus* B-276 is also a member of the class of monooxygenases containing a di-iron centre motif at the active site. Two conserved Glu-X-X-His domains, which provide the protein-derived ligands to the di-iron centres in the soluble methane monooxygenase, were found in each of these large oxygenase subunits (Saeki & Furuhashi, 1994).

We cloned and sequenced the genes encoding the alkene

monooxygenase from *R. corallinus* B-276 (Saeki & Furuhashi, 1994). These studies revealed that the alkene monooxygenase genes are clustered in the order *amoA*, *amoB*, *amoC*, *amoD*, and encode the small subunit of the epoxygenase, the coupling protein, the large subunit of the epoxygenase and the reductase, respectively. A comparison of the amino acid sequence of AmoC with the amino acid sequence of the methane monooxygenase (MmoX) of *Methylococcus capsulatus* (Bath) revealed the presence of two conserved Glu-X-X-His motifs suggested to act as iron cluster ligands, thus participating in a hydrogen-bonding network. Based on the conservation of these amino acids, we proposed that the alkene monooxygenase contains binuclear iron centres, and this was confirmed by the results gained from electromagnetic resonance spectral analysis of the purified epoxygenase component (Gallagher *et al.*, 1997).

In this study, we report on the identification of the alkene monooxygenase in *R. corallinus* B-276 as the TCE-oxidizing enzyme system. TCE degradation was found to be inducer-independent but growth-phase-dependent. We describe the identification of linear plasmids in *R. corallinus* B-276 and provide clear evidence that the alkene monooxygenase is encoded by the linear plasmid pNC30.

METHODS

Bacterial strains and growth conditions. *R. corallinus* B-276 was previously isolated from soil (Furuhashi *et al.*, 1981). The 15 different variants (*R. corallinus* B-276.101–B-276.115) used in this study were obtained after growth with NBG broth containing 2.8% (w/v) nutrient broth no. 2 (Oxoid) and 1.0% (w/v) glucose at 30 °C under nonselective conditions. *R. corallinus* B-276 cells were grown in mineral medium (M3) containing (per litre) 1.0 g NH_4NO_3 , 0.82 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.87 g KH_2PO_4 , 6.4 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 ml trace element solution (Tschech & Pfennig, 1984) and 1 ml vitamin solution (Claus *et al.*, 1983). Propene as a source of carbon was supplied via the gas phase. Incubation in M3 or NBG media was carried out at 30 °C. Cultures were grown either in shake flasks or in sealed shake flasks with propene (10%, v/v) supplied via the gas phase. *E. coli* JM109(pSTT100) and JM109(pUC18) were grown in Luria–Bertani (LB) medium supplemented with 50 µg ampicillin ml^{-1} at 37 °C. To induce the transcription of the alkene monooxygenase genes from the *lac* promoter, IPTG was added to a final concentration of 1.0 mM after 4.5 h incubation and cells were further incubated for 3 h. The strains used in this study are listed in Table 1.

Degradation of chlorinated hydrocarbons. The *R. corallinus* B-276 cells were harvested by centrifugation at 8000 r.p.m. for 10 min at 4 °C, washed once and resuspended in reaction buffer (per litre deionized water: K_2HPO_4 , 1.74 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.5 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mg; pH 7.5). Cell suspensions (5 ml) were transferred to 152 ml glass serum bottles sealed with Teflon-lined Mininert Valves (Dynatech). To perform TCE degradation experiments with recombinant *E. coli* strains, the cells were grown at 37 °C in 400 ml LB medium with 1% glucose in the presence of 50 mg ampicillin ml^{-1} . IPTG was added to a final concentration of 1 mM after 4.5 h of growth. After further incubation for 2.5 h, the cells were harvested, washed and resuspended in 40 ml reaction buffer. The degradation experiments were started by the

Table 1. Bacterial strains used in this study

Strain	Relevant properties*	Size of linear plasmids (kb)†	Source or reference
<i>Escherichia coli</i> JM109(pSTT100)	<i>amoABCD</i> , Ap ^r	NA	Saeki & Furuhashi (1994)
<i>Escherichia coli</i> JM109(pUC18)	Ap ^r	NA	Yanisch-Perron <i>et al.</i> (1985)
<i>Rhodococcus corallinus</i> B-276	Wild-type, PRO ⁺	70, 85, 185, 235	Furuhashi <i>et al.</i> (1981)
Spontaneous mutants of <i>Rhodococcus corallinus</i> B-276			
B-276.101	PRO ⁻	70, 85, 235	This study
B-276.102	PRO ⁻	70, 85, 100, 235	
B-276.103	PRO ⁻	70, 85, 235	
B-276.104	PRO ⁻	70, 85, 235	
B-276.105	PRO ⁻	70, 85, 235	
B-276.106	PRO ⁻	70, 85, 235, 250	
B-276.107	PRO ⁻	70, 85, 235	
B-276.108	PRO ⁻	70, 85, 235	
B-276.109	PRO ⁻	70, 85, 235	
B-276.110	PRO ⁻	70, 85, 235	
B-276.111	PRO ⁻	70, 85, 235	
B-276.112	PRO ⁻	70, 85, 235, 290	
B-276.113	PRO ⁻	70, 85, 235	
B-276.114	PRO ⁻	70, 85, 235	
B-276.115	PRO ⁻	70, 85, 95, 235	
<i>Rhodococcus rhodochrous</i> P-3-28	PRO ⁺	70, 230	K. Furuhashi (unpublished)
<i>Rhodococcus rhodochrous</i> P-3-101-1	PRO ⁺	70, 85, 230	
<i>Rhodococcus ruber</i> P-IV-B-11	PRO ⁺	260	
<i>Rhodococcus ruber</i> P-V-B-171	PRO ⁺	70, 260	
<i>Rhodococcus fascians</i> B-2-2	BUT ⁺	ND	
<i>Rhodococcus fascians</i> B-2-25	BUT ⁺	ND	
<i>Rhodococcus fascians</i> B-3-36-3	BUT ⁺	ND	
<i>Mycobacterium</i> sp. E-2-1	ETH ⁺	ND	
<i>Mycobacterium</i> sp. E-2-47	ETH ⁺	ND	
<i>Mycobacterium</i> sp. E-1-35	ETH ⁺	ND	
<i>Mycobacterium</i> sp. E-1-57	ETH ⁺	260, 340	Dabrock <i>et al.</i> (1994)
<i>Rhodococcus erythropolis</i> BD2	IPB ⁺	208	
<i>Rhodococcus erythropolis</i> BD2.101	IPB ⁻	ND	

NA, Not analysed.

ND, None detected.

* PRO⁺, propene-oxidizing character; BUT⁺, 1,3-butadiene-oxidizing character; ETH⁺, ethene-oxidizing character; IPB⁺, isopropylbenzene-oxidizing character.

† All sizes are approximate.

addition of substrates. TCE and other chlorinated hydrocarbon stock solutions were prepared in completely filled vials by dissolving them in *N,N*-dimethylformamide (DMF). Glucose solution (10%; 250 µl) was added to the cell suspensions before adding TCE. No more than 5 µl stock solutions of chlorinated hydrocarbons per 5 ml cell suspension were added to avoid undesired side-effects of DMF. The bottles were incubated in a water bath at 30 °C with reciprocal shaking at 150 r.p.m. TCE degradation was quantified by headspace gas chromatography as described recently (Berendes *et al.*, 1998). All data given reflect the results of experiments in duplicate

bottles. To determine the K_m values, the actual TCE concentration in the liquid phase was calculated using a Henry's constant of 0.5 at 30 °C (Amoore & Hautala, 1983). The kinetic parameters for TCE degradation, V_{max} and K_m , were determined from the Lineweaver-Burk plot assuming that TCE degradation follows Michaelis-Menten kinetics. The protein content was measured by the method of Schmidt *et al.* (1963).

Degradation of propene. Propene degradation experiments were conducted analogously to the TCE degradation studies (column temperature for GC was changed to 200 °C). Propene

was supplied via the gas phase. To determine the K_m values, the actual propene concentration in the liquid phase was calculated from the Bunsen coefficient for propene at 30 °C (Wilhelm *et al.*, 1977). Kinetic parameters for propene degradation, V_{max} and K_m , were determined from the Lineweaver-Burk plot assuming that propene degradation follows Michaelis-Menten kinetics.

DNA manipulation. DNA manipulations were performed as described by Sambrook *et al.* (1989).

Preparation of DNA for detection of linear plasmids. Cultures of bacterial strains were grown for 24–48 h at 30 °C in LB medium supplemented with 1% glucose and 1% (w/v) glycine. Samples (0.35 ml) of the cultures were centrifuged at 8000 r.p.m. and resuspended in 0.25 ml EET [0.1 M EDTA, 10 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid, 10 mM Tris/HCl, pH 8.0] and then mixed with 0.75 ml 2.0% (w/v) low-melting-point agarose at 40 °C to prepare agarose plugs. The resulting plugs were incubated in TE buffer (50 mM Tris, 20 mM EDTA; pH 8.0) containing lysozyme (2 mg ml⁻¹), *Achromobacter lyticus* peptidase (2 mg ml⁻¹) (Wako Pure Chemicals) and sodium *N*-lauroylsarcosine (0.5 mg ml⁻¹) for 4 h at 30 °C and then treated at 55 °C overnight in EET buffer containing 1% (w/v) SDS. The plugs were washed in TE buffer for 2 h and finally stored in TE buffer at 4 °C until they were used for contour-clamped homogeneous electric field electrophoresis (CHEFE).

CHEFE. This was performed by using a Pulsaphor system obtained from Pharmacia. It was carried out at 15 °C with a switching interval of 10–20 s for 17 h or 10–40 s for 15 h at 170 V in 0.5 × TBE (44.5 mM Tris, 44.5 mM borate, 1 mM EDTA; pH 8.5) using a 0.8% agarose gel. DNA bands were stained with ethidium bromide and visualized by means of a UV-transilluminator. Size determinations of linear plasmids were performed by the use of concatemers of λ DNA as high-molecular-mass DNA standards (Anand, 1986; Matthew *et al.*, 1988).

Southern hybridization. After CHEFE separation, DNA was transferred to nylon membranes (GeneScreen Plus; Dupont, NEN Research Products) using a vacuum blotting system. The *amoABC*-specific DNA probe (a 1.8-kb *Pst*I–*Not*I DNA fragment derived from pSTT100) was treated with a digoxigenin-dUTP DNA labelling kit (Boehringer Mannheim). Hybridization reactions and subsequent detection with a DIG luminescent detection kit (Boehringer Mannheim) were performed according to the manufacturer's protocol. Hybridization reactions were performed at 55 °C and the filters were washed twice with 0.5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate)/0.1% SDS for 30 min and once with 0.1 × SSC/0.1% SDS for 10 min at 55 °C.

RESULTS

Trichloroethene degradation by *R. corallinus* B-276

A propene-grown culture of *R. corallinus* B-276 was harvested after 72 h to study the TCE degradation of resting cells. The ability of strain B-276 to degrade TCE is depicted in Fig. 1(a). Immediately after the addition of TCE, the cells started TCE oxidation. No TCE degradation was detected in a control experiment without cells. To analyse the growth-substrate dependence of TCE oxidation, the TCE degradation of NBG-grown resting cells of strain B-276 was monitored. As depicted

in Fig. 1(b), growth of strain B-276 with glucose led to TCE oxidation rates comparable to those obtained with propene-grown cells. Twenty-five per cent of the initial amount of TCE was degraded within 6 h of incubation.

Growth-phase dependence of TCE degradation by *R. corallinus* B-276

Studies on growth-phase-dependent alkene monooxygenase activity in *R. corallinus* B276 have been performed by Miura (1993) and Furuhashi *et al.* (1981). These studies clearly showed that the alkene monooxygenase activity is induced after 24 h growth on glucose during the onset of stationary phase. In order to evaluate the effect of growth phase on TCE degradation, we monitored the TCE degradation rates of late-exponential-phase cells (24 h growth) and stationary-phase cells (48, 72 or 96 h growth). The growth-phase dependence of TCE degradation is obvious from the data in Fig. 1(b). With late-exponential-phase cells, no TCE degradation was found during the first 6 h of incubation, whereas stationary-phase cells started to oxidize TCE immediately after addition of TCE. No difference in TCE degradation efficiency was detected with resting cells of B-276 cultures grown for 48, 72 and 96 h, respectively (Fig. 1b). Further incubation of the late-exponential-phase cells for more than 6 h led to an increased TCE oxidation capability (35% of the TCE supplied was degraded between 6 and 23 h of incubation). The immediate TCE degradation of B-276 stationary-phase cells together with the correlating results of Miura (1993) showing that alkene monooxygenase is induced after glucose has been consumed, indicate that the expression of the alkene monooxygenase might be controlled by catabolite repression.

Kinetics of TCE and propene degradation

The initial TCE degradation rates by *R. corallinus* B-276 were studied with respect to different TCE concentrations (data not shown). The actual TCE concentration in the liquid phase (30–200 mM) was calculated using a TCE gas/water partition coefficient (concentration in air/concentration in water) of 0.5 at 30 °C (Amoore & Hautala, 1983). The initial TCE degradation rates were determined for the first hour. V_{max} and K_m values calculated from the initial TCE degradation rates during the first hour of incubation were 2.4 nmol min⁻¹ (mg protein)⁻¹ and 187 μ M, respectively. The kinetics of propene degradation were also studied. Taking into consideration the partitioning of propene between the gas and liquid phase by means of the published Bunsen coefficients for gases (Wilhelm *et al.*, 1977), V_{max} and K_m values of propene degradation were calculated to be 59 nmol min⁻¹ (mg protein)⁻¹ and 16.0 μ M, respectively. Taken together, these results show that the maximum degradation rate and the affinity of the alkene monooxygenase of *R. corallinus* B-276 for propene are significantly higher than those for TCE.

Fig. 1. Extent of TCE degradation by *R. corallinus* B-276. The assays were performed with resting cells (OD_{600} 5.0) in the presence of 5 mmol TCE. (a) $\circ$, Cells grown on M3 (propene) for 72 h; $\bullet$, abiotic control. (b) $\square$, $\blacksquare$, $\triangle$, $\blacktriangle$, Cells grown on NBG for 24, 48, 72 and 96 h, respectively; $\bullet$, abiotic control.

Fig. 2. Time course of the degradation of chlorinated solvents by resting cells of *R. corallinus* B-276. The assays were performed in the presence of 2 mmol chlorinated solvent with NBG-grown cells (OD_{600} 5.0) as described in Methods. Symbols on solid lines: $\circ$, TCE; $\bullet$, 1,1-dichloroethene; $\square$, *trans*-1,2-dichloroethene; $\blacksquare$, *cis*-1,2-dichloroethene; $\triangle$, 2,3-dichloropropene; $\blacktriangle$, 1,3-dichloropropene. Symbols on broken lines: $\circ$, dichloromethane; $\bullet$, 1,2-dichloromethane; $\square$, chloroform.

Substrate specificity of the alkene monooxygenase

Cell suspensions of *R. corallinus* B-276 were tested for their ability to degrade different chlorinated compounds. As depicted in Fig. 2, the resting cells started to degrade TCE, 1,1-dichloroethene, *trans*-1,2-dichloroethene, *cis*-1,2-dichloroethene, 2,3-dichloropropene and 1,3-dichloropropene immediately after the addition of the corresponding chlorocarbon compound. Among the chlorinated alkenes degraded, *cis*-1,2-dichloroethene and 2,3-dichloropropene were almost completely consumed within 4 h; 83% of 1,1-dichloroethene and 81%

of 1,3-dichloropropene were degraded within 23 h of incubation, whilst only about 38% of TCE and 37% of *trans*-1,2-dichloroethene were degraded within 23 h of incubation. Chlorinated alkanes, including dichloromethane, 1,2-dichloroethane and chloroform, were not converted by strain B-276. As shown by studies with a spontaneous mutant *R. corallinus* B-276.101, the loss of the ability to grow on propene correlated with the loss of the ability to degrade chlorinated alkenes.

Alkene-monooxygenase-mediated TCE degradation by recombinant *E. coli* JM109 cells

By analogy with the alkene monooxygenase of *Xanthobacter* Py2, we suggested that the alkene monooxygenase in strain B-276 was responsible for the cooxidation of TCE. To address this question, the TCE degradation of the recombinant strain *E. coli* JM109(pSTT100), which contains the structural genes of the alkene monooxygenase of *R. corallinus* B-276 under the control of the *lac* promoter, was investigated. The expression of the alkene monooxygenase genes in *E. coli* JM109(pSTT100) in response to the addition of IPTG has already been shown by Saeki & Furuhashi (1994). TCE degradation studies of recombinant *E. coli* strains revealed that 35% of the total TCE supplied was degraded by the alkene-monooxygenase-carrying strain *E. coli* JM109(pSTT100) after 16 h of incubation. No TCE degradation was found in the controls, such as with heat-killed *E. coli* cells and recombinant *E. coli* cells carrying pUC18. These results strongly suggest that the alkene monooxygenase of *R. corallinus* B-276 is mediating TCE oxidation.

Detection of linear megaplasms in *R. corallinus* B-276

When we examined the propene degradation of *R. corallinus* B-276, we observed a spontaneous instability of the propene-oxidizing phenotype (PRO⁺). Revertants from PRO⁻ to PRO⁺ were not observed. These results

Fig. 3. Detection of linear DNA molecules from *R. corallinus* B-276 by CHEFE. CHEFE was carried out at 170 V for 17 h with 10–20 s pulse times. Lanes: 1, λ ladder; 2–4, agarose-embedded B-276 DNA treated with lysis buffer containing (per ml TE buffer) 2.0 mg lysozyme, 0.5 mg *N*-lauroylsarcosine and 2 mg (lane 2), 1 mg (lane 3) or 0.5 mg (lane 4) *A. lyticus* peptidase.

strongly suggest that the genes responsible for propene degradation of *R. corallinus* B-276 are localized on a mobile DNA element. Recent studies revealed that large linear plasmids of nocardioform actinomycetes are involved in catabolic functions including oxygenases such as isopropylbenzene dioxygenase (Dabrock *et al.*, 1994) and biphenyl dioxygenase (Masai *et al.*, 1997). To analyse the propene-oxidizing wild-type strain B-276 for linear plasmids, we performed CHEFE with agarose-embedded lysed B-276 cells. These analyses led to the identification of four different linear plasmids named pNC10 (70 kb), pNC20 (85 kb), pNC30 (185 kb) and pNC40 (235 kb) (Fig. 3, lane 2). To confirm the linear topology of these plasmids, separate gels were run with different pulse times. During CHEFE, the extent of migration of circular molecules relative to linear molecules varies considerably according to pulse times (Saeki & Furuhashi, 1994). All four plasmids were confirmed to be linear based on the relative migration positions compared to the reference λ ladder at different pulse times (data not shown). When the concentration of *A. lyticus* peptidase in the lysis buffer was reduced from 2 to 1 or 0.5 mg ml⁻¹, three of the four linear plasmids, pNC20, pNC30 and pNC40, became nondetectable (Fig. 3). The detection of pNC10 remained unaffected.

Analysis of linear plasmids in spontaneous mutants which had lost the ability to grow on propene

A total of fifteen spontaneous mutants which had lost the ability to grow on propene (PRO⁻ variants) (Table 1) were analysed by CHEFE for the presence of linear plasmids. All mutants lacked plasmid pNC30 (Fig. 4a). This observation strongly suggests that either part or even the complete propene degradation pathway is

Fig. 4. Detection of linear DNA molecules in *R. corallinus* strains. (a) CHEFE analysis of *R. corallinus* B-276 and spontaneous mutants which had lost the ability to grow on propene; (b, c) Southern blot analysis using the DIG-labelled 1.8 kb *Pst*I–*Not*I fragment of plasmid pSTT100 carrying the alkene monooxygenase genes of *R. corallinus* B-276. CHEFE was carried out at 170 V for 17 h with 10–20 s pulse times. (a) Lanes: 1, λ ladder; 2, *R. corallinus* B-276; 3–17, *R. corallinus* B-276.101 to B-276.115. (b, c) Lanes: 1, *R. corallinus* B-276; 2, *R. corallinus* B-276.101; 3, *R. corallinus* B-276.102; 4, *R. corallinus* B-276.106; 5, *R. corallinus* B-276.112.

encoded by the linear plasmid pNC30. Among the PRO⁻ variants, two strains contained an additional linear plasmid of smaller size and two other strains contained an additional linear plasmid of larger size with a simultaneous loss of pNC40 (Table 1, Fig. 4a). The linear plasmid of smaller size is most probably a deletion derivative of pNC30 (pNC30 Δ), and the linear plasmid of larger size might be a recombinant plasmid of pNC30 Δ and pNC40.

Localization of the alkene monooxygenase genes on the linear plasmid pNC30

To analyse the localization of the alkene monooxygenase genes, a Southern hybridization was performed with a 1.8 kb *Pst*I–*Not*I fragment carrying alkene

monooxygenase genes of *R. corallinus* B-276. For these hybridizations, linear plasmid DNA of the PRO⁺ B-276 wild-type strain, of the spontaneous PRO⁻ mutant B-276.101 and of three other spontaneous PRO⁻ mutants, B-276.102, B-276.106 and B-276.112, which contain additional large or small plasmids, was used. The hybridization profile observed with undigested linear plasmid DNA revealed hybridization signals with a 185 kb linear DNA fragment and with the chromosomal DNA. The 185 kb signal corresponds to pNC30 (Fig. 4b, c). No signals were detected with *Pst*I-digested total DNA isolated from PRO⁻ mutants devoid of pNC30 (data not shown). The lack of any hybridization signal of the alkene monooxygenase genes with DNA from the PRO⁻ mutant suggests that the signals with the main bacterial chromosome (Fig. 4c) result from unspecific hybridizations due to very high amounts of chromosomal DNA separated in the CHEFE gels. These results, together with the PRO⁻ phenotype of the pNC30 mutant, provide clear evidence that the alkene monooxygenase genes are located on the linear plasmid pNC30. Further analysis of PRO⁻ variants revealed that these variants had also lost the capacity to degrade propene oxide, which is an intermediate of propene degradation (data not shown). These results suggest that in addition to the alkene monooxygenase the linear plasmid pNC30 encodes enzymes implicated in the further breakdown of the propene oxide produced from propene by the alkene monooxygenase in *R. corallinus* B-276.

Survey of linear plasmids from short-chain-alkene-oxidizing strains

Stock cultures able to grow on either ethene, propene or 1,3-butadiene have been isolated from various soil samples employing conventional enrichment and isolation techniques. Among the strains isolated with propene as sole source of carbon, four showed the ability to epoxidize propene after growth in NBG medium. These strains were identified by the National Collection of Industrial and Marine Bacteria (Aberdeen, UK) as *Rhodococcus rhodochrous* P-3-28, P-3-101-1, *Rhodococcus ruber* P-IV-B-11 and P-V-B-171 (Table 1). The strains isolated with ethene or 1,3-butadiene showed either no ability to epoxidize propene after growth in NBG medium or were not able to grow in this medium. Three of the strains isolated with 1,3-butadiene were identified as *Rhodococcus fascians* B-2-2, B-2-25 and B-3-36-3, whereas the four ethene-oxidizing strains, found to be members of the genus *Mycobacterium*, were designated *Mycobacterium* sp. E-2-1, E-2-47, E-1-35 and E-1-57.

The newly isolated strains were analysed for the presence of linear plasmids. All propene-oxidizing strains and one ethene-oxidizing strain were found to contain linear plasmids of different sizes (Table 1). These findings support the suggestion that linear plasmids are broadly distributed in a wide range of actinomycetes.

Fig. 5. Linear DNA molecules in lysed cells of propene-oxidizing *Rhodococcus* strains and an ethene-oxidizing *Mycobacterium* strain by CHEFE and Southern blot analysis. (a) CHEFE was carried out at 170 V for 15 h with 10–40 s pulse times; (b) a DIG-labelled 1.8 kb *Pst*I–*Not*I fragment of plasmid pSTT100 carrying alkene monooxygenase genes of *R. corallinus* B-276 was used as probe. Lanes: 1, λ ladder; 2, *R. corallinus* B-276; 3, *R. rhodochrous* P-3-28; 4, *R. rhodochrous* P-3-101-1; 5, *R. ruber* P-IV-B-11; 6, *R. ruber* P-V-B-171; 7, *Mycobacterium* sp. E-1-57.

Distribution of plasmid-borne alkene monooxygenases in different *Rhodococcus* species

To analyse the distribution of linear plasmids carrying alkene monooxygenase genes, Southern hybridizations were performed using an *amoABC* probe (the 1.8 kb *Pst*I–*Not*I fragment from pSTT100). These studies revealed that the alkene monooxygenase genes from strain B-276 hybridized to the 260 kb linear plasmids of the propene-oxidizing strains *R. ruber* P-IV-B-11 and P-V-B-171 (Fig. 5b). These results suggest the presence of similar alkene monooxygenase genes on these linear plasmids of the two *R. ruber* strains.

DISCUSSION

In this study, we report on the TCE degradation of the alkene monooxygenase from *R. corallinus* B-276 and provide clear evidence that the alkene monooxygenase of strain B-276 is located on a linear megaplasmid. To our knowledge, this is the first report of the detection of a linear plasmid encoding alkene degradation.

With the exception of *N. europaea*, TCE-degrading wild-type strains require the addition of exogenous inducer substrates for induction of TCE-cooxidizing oxygenases (Ensley, 1991). Usually, physiological substrates of such oxygenases serve as inducer substrates, although there have been reports of TCE-mediated induction of TCE-cooxidizing toluene mono- or dioxygenases in *Pseudomonas* strains (McClay *et al.*, 1995; Leahy *et al.*, 1996; Shingleton *et al.*, 1998). Furthermore,

Table 2. Kinetic parameters of TCE degradation by different monooxygenases

Micro-organism	Enzyme	Growth substrate	$V_{\max}$ [nmol min ⁻¹ (mg protein) ⁻¹]	K_m (μ M)	Reference
<i>Rhodococcus corallinus</i> B-276	Alkene monooxygenase	NBG medium	2.4	187	This study
<i>Xanthobacter</i> sp. Py2	Alkene monooxygenase	Propene	16	116	Reij <i>et al.</i> (1995)
<i>Burkholderia cepacia</i> G4	Toluene 2-monooxygenase	Phenol	9	4	Sun & Wood (1996)
<i>Burkholderia cepacia</i> G4 PR1	Toluene 2-monooxygenase	LB medium	18	29	Sun & Wood (1996)
<i>Pseudomonas mendocina</i> KR1	Toluene 3-monooxygenase	Phenol	20	10	Sun & Wood (1996)
<i>Pseudomonas putida</i> F1	Toluene dioxygenase	Toluene	8	5	Sun & Wood (1996)
<i>Methylosinus trichosporium</i> OB3b	Methane monooxygenase	Methane	509	145	Sun & Wood (1996)

it has been shown that a range of aliphatic and chlorinated alkenes and epoxides serve as inducers for alkene monooxygenase and epoxidase expression in *Xanthobacter* Py2 (Ensign, 1996). The physiological substrates of the TCE-cooxidizing oxygenases are competitive substrates for the TCE degradation enzymes and some compounds are toxic, which may preclude their environmental use. To overcome these intrinsic obstacles of the wild-type strains, selection of strains exhibiting inducer-independent TCE degradation capabilities or the construction of recombinant *Pseudomonas* strains in which TCE-oxidizing enzymes are under the control of an alternative promoter have been performed. For example, *B. cepacia* G4PR1 constitutively expressing toluene-2-monooxygenase has been derived from *B. cepacia* G4 (Shields & Reagin, 1992) and *Pseudomonas* hybrid strains (JR1A::ipb and CBS-3::ipb) constitutively expressing isopropylbenzene dioxygenase have been constructed (Berendes *et al.*, 1998).

In contrast to other strains degrading TCE, alkene monooxygenase-mediated TCE degradation by *R. corallinus* B-276 occurred in a growth-phase-dependent manner independent of inducer substrates, such as the physiological substrates of the alkene monooxygenase or chlorinated alkenes. Accordingly, glucose as carbon source led to TCE degradation rates comparable to those obtained with propene-grown cultures. To our knowledge, this is the first TCE-degrading wild-type strain neither requiring physiological substrates of oxygenases nor chlorinated alkenes to achieve expression of the TCE-oxidizing enzyme system. However, the presence of unknown inducers or repressors in NBG medium cannot be ruled out. A thorough understanding of how alkene monooxygenase is regulated will require the identification and characterization of regulatory genes, which are currently being performed.

Besides TCE, a number of other chlorinated compounds were degraded by NBG-grown cells of *R. corallinus* B-276. All chlorinated alkenes tested were at least partially oxidized whereas chlorinated alkanes tested were not degraded. These results are consistent with known properties of the alkene monooxygenase enzyme system, which does not exhibit alkane hydroxylase activity. Propene-grown cells of *Xanthobacter* Py2 have also

been shown not to degrade chlorinated alkanes (Ensign *et al.*, 1992; Ensign, 1996). Although the substrate specificity of the alkene monooxygenases of *R. corallinus* B-276 and *Xanthobacter* Py2 was comparable with respect to chlorinated alkenes, there were significant differences in degradation efficiencies, for example 1,1-dichloroethene was the poorest substrate for the alkene monooxygenase of *Xanthobacter* Py2 among the chlorinated alkenes tested but this compound was more efficiently degraded by *R. corallinus* B-276 than TCE, *trans*-1,2-dichloroethene and 1,3-dichloropropene.

The apparent K_m and $V_{\max}$ values for TCE degradation by *R. corallinus* B-276 were determined. Comparison of the kinetic parameters of NBG-grown *R. corallinus* B-276 with those of propene-grown *Xanthobacter* Py2, phenol-grown *B. cepacia* G4, LB medium-grown *B. cepacia* G4PR1, phenol-grown *P. mendocina* KR1, toluene-grown *P. putida* F1 and methane-grown *Methylosinus trichosporium* OB3b revealed that the $V_{\max}$ value of *R. corallinus* B-276 was quite low and the K_m value was at the highest level (Table 2). The kinetic parameters for propene indicate that propene is a much better substrate for the alkene monooxygenase than TCE. The applicability of *R. corallinus* B-276 for TCE degradation in the environment might be restricted by the low affinity and low degradation rates of the alkene monooxygenase, but the advantage of this enzyme system is that glucose-grown resting cells, which are easy to prepare, can be used for a TCE bioremediation process.

During our studies, we found that *R. erythropolis* BD2, which carries the linear plasmid pBD2 encoding an isopropylbenzene-degradation pathway (Dabrock *et al.*, 1994), has the ability to grow on propene as a sole carbon and energy source. A spontaneous mutant strain BD2.101, which is pBD2-deficient, lost the ability to grow on propene (data not shown). These results indicate that the genes responsible for the propene catabolism of strain BD2 are also localized on the linear plasmid pBD2. However, no hybridization signal was detected with the plasmid pBD2 or *Pst*I-digested total DNA with the probe of alkene monooxygenase genes from *R. corallinus* B-276 (data not shown). This leads to the suggestion that the *R. erythropolis* BD2 genes responsible for the growth on propene differ from the

alkene monooxygenase genes in *R. corallinus* B-276 and might even encode an enzyme in a different family.

Southern hybridization of *amoABC* genes with linear plasmid DNA of the short-chain-alkene-oxidizing strains revealed specific signals with the 260 kb linear plasmids of two propene-oxidizing *R. ruber* strains. These results suggest that the *R. ruber* strains carry linear plasmids encoding alkene monooxygenases which exhibit significant similarities to the alkene monooxygenase of *R. corallinus* B-276. Taken together, our results strengthen the hypothesis that linear plasmids play a major role in acquiring novel catabolic capabilities by horizontal gene transfer between *Rhodococcus* species and therefore can be considered to be important tools in the evolution of biochemical pathways.

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REFERENCES

- Amoore, J. E. & Hautala, E. (1983). Odor as an aid to chemical safety: odor threshold values compared with threshold limit values and volatilities for 214 industrial chemicals in air and water dilution. *J Appl Toxicol* 3, 272–290.
- Anand, R. (1986). Pulsed field gel electrophoresis: a technique for fractionating large DNA molecules. *Trends Genet* 2, 278–283.
- Arciero, D., Vannelli, T., Logan, M. & Hooper, A. B. (1989). Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Biochem Biophys Res Commun* 159, 640–643.
- Berendes, F., Sabarth, N., Averhoff, B. & Gottschalk, G. (1998). Construction and use of an *ipb* DNA module to generate *Pseudomonas* strains with constitutive trichloroethene and isopropylbenzene oxidation activity. *Appl Environ Microbiol* 60, 855–860.
- Brusseau, G. A., Tsien, H.-C., Hanson, R. S. & Wackett, L. P. (1990). Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. *Biodegradation* 1, 19–29.
- Claus, D., Lack, P. & Neu, B. (1983). *Deutsche Sammlung von Mikroorganismen und Zellkulturen Catalogue of Strains*, 3rd edn. Braunschweig, Germany: Deutsche Sammlung von Mikroorganismen und Zellkulturen.
- Dabrock, B., Riedel, J., Bertram, J. & Gottschalk, G. (1992). Isopropylbenzene (cumene) – a new substrate for the isolation of trichloroethene-degrading bacteria. *Arch Microbiol* 158, 9–13.
- Dabrock, B., Keßeler, M., Averhoff, B. & Gottschalk, G. (1994). Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethene catabolism. *Appl Environ Microbiol* 60, 853–860.
- Ensign, S. A. (1996). Aliphatic and chlorinated alkenes and epoxides as inducers of alkene monooxygenase and epoxidase activities in *Xanthobacter* strain Py2. *Appl Environ Microbiol* 62, 61–66.
- Ensign, S. A., Hyman, M. R. & Arp, D. J. (1992). Cometary degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown *Xanthobacter* strain. *Appl Environ Microbiol* 58, 3038–3046.
- Ensley, B. D. (1991). Biochemical diversity of trichloroethylene metabolism. *Annu Rev Microbiol* 45, 283–299.
- Fox, B. G., Borneman, J. G., Wackett, L. P. & Lipscomb, J. D. (1990). Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry* 29, 6419–6427.
- Furuhashi, K. (1992). Biological routes to optically active epoxides. In *Chirality in Industry*, pp. 167–186. Edited by A. N. Collins, G. N. Sheldrake & J. Crosby. Chichester & New York: Wiley.
- Furuhashi, K., Taoka, A., Uchida, S., Karube, I. & Suzuki, S. (1981). Production of 1,2-epoxyalkanes from 1-alkenes by *Nocardia corallina* B-276. *Eur J Appl Microbiol Biotechnol* 12, 39–45.
- Gallagher, S. C., Cammack, R. & Dalton, H. (1997). Alkene monooxygenase from *Nocardia corallina* B-276 is a member of the class of dinuclear iron proteins capable of stereospecific epoxidation reactions. *Eur J Biochem* 247, 635–641.
- Gallagher, S. C., George, A. & Dalton, H. (1998). Sequence-alignment modelling and molecular docking studies of the epoxidase component of alkene monooxygenase from *Nocardia corallina* B-276. *Eur J Biochem* 254, 480–489.
- Harker, A. R. & Kim, Y. (1990). Trichloroethylene degradation by two independent aromatic-degrading pathways in *Alcaligenes eutrophus* JMP134. *Appl Environ Microbiol* 56, 1179–1181.
- Kaphammer, B., Kukor, J. J. & Olsen, R. H. (1990). Cloning and characterization of a novel toluene degradative pathway from *Pseudomonas pickettii* PKO1. In *Abstracts of the 90th Annual Meeting of the American Society for Microbiology*, abstract K-145, p. 243. Washington, DC: American Society for Microbiology.
- Kesseler, M., Dabbs, E. R., Averhoff, B. & Gottschalk, G. (1996). Studies on the isopropylbenzene 2,3-dioxygenase and the 3-isopropylcatechol 2,3-dioxygenase genes encoded by the linear plasmid of *Rhodococcus erythropolis* BD2. *Microbiology* 142, 3241–3251.
- Kim, Y., Ayoubi, P. & Harker, A. R. (1996). Constitutive expression of the cloned phenol hydroxylase gene(s) from *Alcaligenes eutrophus* JMP134 and concomitant trichloroethylene oxidation. *Appl Environ Microbiol* 62, 3227–3233.
- Koh, S.-C., Bowman, J. P. & Saylor, G. S. (1993). Soluble methane monooxygenase production and trichloroethylene degradation by a type I methanotroph, *Methylobomonas methanica* 68-1. *Appl Environ Microbiol* 59, 960–967.
- Leahy, J. G., Byrne, A. M. & Olsen, R. H. (1996). Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. *Appl Environ Microbiol* 62, 825–833.
- McClay, K., Streger, S. H. & Steffan, R. J. (1995). Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas* sp. strain ENVPC5 by chlorinated solvents and alkanes. *Appl Environ Microbiol* 61, 3479–3481.
- Masai, E., Sugiyama, K., Iwashita, N., Shimizu, S., Hauschild, J. E., Hatta, T., Kimbara, K., Yano, K. & Fukuda, M. (1997). The *bphDEF* meta-cleavage pathway genes involved in biphenyl/poly-chlorinated biphenyl degradation are located on a linear plasmid and separated from the initial *bphACB* genes in *Rhodococcus* sp. strain RHA1. *Gene* 187, 141–149.
- Matthew, M. K., Hui, C.-F., Smith, C. L. & Cantor, C. R. (1988). High resolution separation and accurate size determination in pulsed field gel electrophoresis of DNA. IV. The influence of DNA topology. *Biochemistry* 27, 9222–9226.
- Miura, A. (1993). *Purification and characterization of the alkene*

monooxygenase from *Nocardia corallina* B-276. PhD thesis, University of Warwick.

Miura, A. & Dalton, H. (1995). Purification and characterization of the alkene monooxygenase from *Nocardia corallina* B-276. *Biosci Biotechnol Biochem* **59**, 853–859.

Newman, L. M. & Wackett, L. P. (1997). Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. *J Bacteriol* **179**, 90–96.

Oldenhuis, R., Vink, R. L. J. M., Janssen, D. B. & Witholt, B. (1989). Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. *Appl Environ Microbiol* **55**, 2819–2826.

Reij, M. W., Kieboom, J., de Bont, J. A. M. & Hartmans, S. (1995). Continuous degradation of trichloroethylene by *Xanthobacter* sp. strain Py2 during growth on propene. *Appl Environ Microbiol* **61**, 2936–2942.

Saeki, H. & Furuhashi, K. (1994). Cloning and characterization of a *Nocardia corallina* B-276 gene cluster encoding alkene monooxygenase. *J Ferment Bioeng* **78**, 399–406.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schmidt, K., Liaanen-Jensen, S. & Schlegel, H. G. (1963). Die Carotinoide der *Thiorhodaceae*. *Arch Mikrobiol* **46**, 117–126.

Shields, M. S. & Reagin, M. J. (1992). Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. *Appl Environ Microbiol* **58**, 3977–3983.

Shields, M. S., Montgomery, S. O., Chapman, P. J., Cuskej, S. M. & Pritchard, P. H. (1989). Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. *Appl Environ Microbiol* **55**, 1624–1629.

Shingleton, J. T., Applegate, B. M., Nagel, A. C., Bienkowski, P. R. & Sayler, G. S. (1998). Induction of the *tod* operon by trichloroethylene in *Pseudomonas putida* TVA8. *Appl Environ Microbiol* **64**, 5049–5052.

Small, F. J. & Ensign, S. A. (1997). Alkene monooxygenase from *Xanthobacter* strain Py2. *J Biol Chem* **272**, 24913–24920.

Sun, A. K. & Wood, T. K. (1996). Trichloroethylene degradation and mineralization by pseudomonads and *Methylosinus trichosporium* OB3b. *Appl Microbiol Biotechnol* **45**, 248–256.

Tschech, A. & Pfennig, N. (1984). Growth yield increase linked to caffeate reduction in *Acetobacterium woodii*. *Arch Microbiol* **137**, 163–167.

Tsien, H.-C., Brusseau, G. A., Hanson, R. S. & Wackett, L. P. (1989). Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. *Appl Environ Microbiol* **55**, 3155–3161.

Vannelli, T., Logan, M., Arciero, D. M. & Hooper, A. B. (1990). Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Appl Environ Microbiol* **56**, 1169–1171.

Wackett, L. P. & Gibson, D. T. (1988). Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. *Appl Environ Microbiol* **54**, 1703–1708.

Wackett, L. P., Brusseau, G. A., Householder, S. R. & Hanson, R. S. (1989). Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. *Appl Environ Microbiol* **55**, 2960–2964.

Wilhelm, E., Battino, R. & Wilcock, R. J. (1977). Low-pressure solubility of gases in liquid water. *Chem Rev* **77**, 219–262.

Winter, R. B., Yen, K.-M. & Ensley, B. D. (1989). Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. *Bio/Technology* **7**, 282–285.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Zylstra, G. J., Wackett, L. P. & Gibson, D. T. (1989). Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. *Appl Environ Microbiol* **55**, 3162–3166.

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