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Production of chiral epoxides by an ethene-utilising *Micrococcus* sp.

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SUMMARY

An ethene-utilising bacterium was isolated in pure culture from soil and was tentatively identified as a *Micrococcus* sp. The organism accumulated epoxyalkanes (0.2–13 mM) from internal, terminal, cyclic and aryl-substituted olefins and exhibited a substrate specificity which was different from that expected on the basis of the chemical reactivity pattern in peracid epoxidations. Epoxyalkanes were hydrolysed at a much slower rate than the epoxidation step which allowed them to accumulate. Ethene-grown cells catalysed the stereospecific formation of R-1,2-epoxypropane (enantiomeric excess: e.e. = 96%), R-1,2-epoxybutane (e.e. = 94%) and *trans*-(2R,3R)-epoxybutane (e.e. = 84%). An ethene monooxygenase was implicated in the production of chiral epoxides in cell-free extracts of the bacterium. The (2S,3S)-enantiomer of racemic *trans*-2,3-epoxybutane was stereoselectively hydrolysed to completion resulting in an enrichment in the (2R,3R)-enantiomer. Further hydrolysis of 1,2-epoxyalkanes (C_3–C_4), however, occurred via complete destruction of both stereoisomers.

INTRODUCTION

Epoxides are useful starting materials in organic synthesis and are used as precursors in the synthesis of a variety of agrochemical and pharmaceutical compounds [18]. Selective direct oxygenation of organic substrates remains a largely unresolved challenge to the synthetic chemist. No general organic chemical method is yet available for the selective synthesis of enantiomeric forms of epoxides, with the notable exception of asymmetric epoxidation of allylic alcohols [26] and the chiral formation of 1,2-epoxypropane [17]. Biological systems are, however, capable of stereoselective epoxidation of prochiral olefins, due to the involvement of monooxygenases. Bacterial production of epoxides from aliphatic olefins has been extensively investigated during the past decade, often with the objective of exploring the possible biotechnological applications. Several epoxide-producing systems have been patented [8,11,24,25,27,29].

Gaseous alkane-utilising bacteria are generally not suitable as chiral epoxide producers since the epoxyalkanes are usually obtained in a very low optical purity [38]. Bacteria which are able to grow only on olefins (and not alkanes) form epoxyalkanes stereospecifically, but the enantiomeric composition depends on both the organism used and the olefin oxidised. In general, epoxyalkanes are formed from non-growth supporting alkenes [15]. Despite the stereospecificity of microbial epoxidations, production of chiral epoxides on a preparative scale is often hindered because the majority of gaseous alkene-utilising bacteria also contain an epoxide-degrading enzyme(s) that hydrolyses the product at a much faster rate than the epoxidation step, thus preventing any significant accumulation of epoxyalkanes [12,13,15,19]. Only a few genera, with a limited substrate specificity, have been isolated on gaseous olefins. These include *Mycobacterium* spp. [4,8,9,15,21], *Nocardia* spp. [10,14,16,19,37], *Xanthobacter* spp. [12] and a Gram-negative motile rod [6].

In this paper, we report on the substrate specificity, enantioselectivity of epoxide degradation and the involvement of an alkene monooxygenase in the production of chiral epoxides by the newly isolated ethene-utilising *Micrococcus* sp.

MATERIALS AND METHODS

Chemicals

Gaseous alkanes/alkenes (research grade) were obtained from British Oxygen Co., London, UK. Epoxides,

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liquid alkanes/alkenes and other chemicals (Gold label) were obtained from Aldrich Chemical Co. Ltd. Gillingham, Dorset, UK. Bacteriological media and reagents for characterisation tests and staining materials (Gold label), were obtained from Difco Laboratories, East Molesey, Surrey, UK. Common chemicals (AR grade), dyes and indicators were obtained from BDH Chemicals Ltd., Poole, Dorset, UK.

Culture isolation

Approximately 1 g of inoculum (soil, mud) was added to 25 ml of basal liquid medium in 250-ml flasks sealed with Suba-seal caps (Freeman & Co., Barnsley, UK). Ethene (5% v/v) was injected into each flask. The liquid was turbid after 5–7 weeks of stationary incubation. Plates of basal medium were spread with serial dilutions of material taken from each flask and incubated at 30 °C in dessicators with ethene at 5% (v/v). Plates were examined for colony formation at 4-day intervals over a period of 3–5 weeks. The colonies were picked and streaked onto basal medium, solidified with purified agar, and incubated for a further 2–3 weeks with ethene. Single colonies were picked and re-streaked onto fresh basal medium and nutrient agar plates to ensure the purity of the culture. Strain M90C was selected for its ability to grow on a range of gaseous alkenes (5% v/v) as the sole source of carbon and energy and was tentatively identified as a *Micrococcus* sp. [2].

Maintenance and growth conditions

Pure cultures of the bacterium were kept on substrate plates at 4 °C and subcultured regularly. Longer term maintenance was on nutrient agar slopes at 4 °C or by storage in glycerol (10% w/v) at −40 °C. The basal mineral-salts (BMS) medium was of the following composition (per litre of distilled water): $Fe_2SO_4 \cdot 7H_2O$ (1 mg); $CuSO_4 \cdot 5H_2O$ (5 μg); H_3BO_3 (10 μg); $ZnSO_4 \cdot 7H_2O$ (70 μg); $MnCl_2 \cdot 4H_2O$ (13 μg); $MgSO_4 \cdot 7H_2O$ (0.2 g); $CaCl_2 \cdot 2H_2O$ (20 mg); $NaMoO_4 \cdot 2H_2O$ (0.1 ml of 0.1% w/v stock solution); KNO_3 (2.4 g); Bacto agar (1.6% w/v) for solid medium. Sterile phosphate buffer (pH 7.2) was added after autoclaving to a final concentration of 25 mM.

Growth on different carbon sources was determined in 250-ml Erlenmeyer flasks containing 50 ml BMS medium with the appropriate substrate. The concentration of the gaseous hydrocarbons was set at 5% (v/v) by injecting the gas in a known volume. Other carbon sources were supplied at either 0.2% (v/v) or 0.5% (w/v). Larger scale cultivation was carried out in 2-l flasks containing 400 ml BMS medium with the gaseous olefins. The inoculum (1–4% v/v) consisted of cells pre-adapted on each substrate.

Bioconversions

Cells were harvested during exponential growth by centrifugation (10800 × *g* at 4 °C for 30 min), washed twice with 25 mM phosphate buffer (pH 7) and resuspended in fresh buffer. Washed-cell suspensions of the bacterium were pre-incubated for 5 min in either 7-ml stoppered flasks (for gaseous hydrocarbons) containing 1 ml of resting cells or in 100-ml screw-capped conical flasks (for liquid hydrocarbons) containing 5–10 ml of resting cells. The bioconversion was started by injecting gaseous olefins (40% v/v) to displace an equal volume of air, whereas liquid olefins were added to a final concentration of 2 mM. All incubations were carried out in triplicate at 30 °C in a shaking water bath at 300 rpm (New Brunswick Scientific Co., Edison, NJ). Control experiments were done with heat-killed cells (by heating at 80 °C for 15 min) and also with reaction mixtures lacking oxygen or substrate.

Preparation of cell-free extracts

Ethene-grown resting cells of *Micrococcus* sp. M90C were divided into two aliquots, with or without the addition of β-mercaptoethanol (5 mM). These were disrupted by two passages through a French pressure cell (American Instruments Co., Silver Spring, MD) at 20000 psi and then centrifuged at 7700 × *g* for 20 min (4 °C) to remove unbroken bacteria and cell debris. The supernatant solution (crude extract) was then centrifuged at 48000 × *g* for 90 min (4 °C), yielding particulate and soluble fractions. The particulate fractions were washed and resuspended in a minimal volume of phosphate buffer (25 mM, pH 7). The fresh subcellular fractions were assayed for their ability to epoxidise a range of gaseous (C_2–C_4) olefins. Assays were carried out in triplicate at 30 °C in 7-ml stoppered flasks (1 ml working volume) containing 0.95 ml of cell-free extracts and 5 μmol of NADH. In those reactions without NADH, 1 ml of cell-free extracts was used. The bioconversion was started by injecting gaseous olefins (40% v/v).

Analytical methods

For C_2–C_4 epoxides, aqueous samples (1 μl) were directly analysed (without extraction) by gas chromatography (Philips, PU 4500) on a glass column packed with Porapak Q (0.9 m × 4 mm i.d.) at 160 °C with the nitrogen carrier gas flow rate at 40 ml/min. For C_2–C_{16} epoxides, aqueous samples (50 μl) were periodically removed from reaction flasks and extracted into an equal volume of ice-cold ether containing 1-alkenes as internal standards. This was shaken vigorously for 1 min, centrifuged at 13000 × *g* for 10 min, cooled, and samples (1 μl) taken from the top ether layer for analysis. The analytical conditions were as follows: Octene: column, 3% SE-30 on Chromosorb W-HP (2.1 m × 4 mm i.d.); temperature, 40 °C (5 min) 12 °C/min to 190 °C; internal standard, 1-nonene.

1-Hexadecene: column, 3% SE-30 on Chromosorb W-HP (2.1 m × 4 mm i.d.); temperature, 180 °C; internal standard, 1-heptadecene. Cyclopentene: column, 10% Carbowax 20M on Chromosorb W-HP (1.8 m × 4 mm i.d.); temperature, 70 °C (5 min) 16 °C/min to 150 °C; internal standard, 1-hexadecene. Cyclohexene: column, 5% OV-17 on Chromosorb W-HP (1.5 m × 4 mm i.d.); temperature, 40 °C (5 min) 16 °C/min to 100 °C; internal standard, 1-nonene. Styrene: column, 10% Carbowax 20M on Chromosorb W-HP (1.8 m × 4 mm i.d.); temperature, 160 °C; internal standard, 1-heptadecene. Phenyl allyl ether: column, 10% Carbowax 20M on Chromosorb W-HP (1.8 m × 4 mm i.d.); temperature, 190 °C; internal standard, 1-heptadecene. Epoxyalkanes were identified by retention time comparisons, and co-chromatography with authentic standards. Identification of the microbially produced epoxides was confirmed by treatment of the reaction mixture with 10–20 μl of concentrated H_2SO_4, followed by gas chromatography, to establish the presence or absence of the epoxide peak after acid hydrolysis [28].

The enantiomeric composition of microbially produced epoxides (C_3–C_4) was determined by chiral complexation gas chromatography [32]. Gaseous samples (100–200 μl) were removed using a gas-tight syringe (Precision Sampling Corp., USA). Analyses were done on a Packard model 436 gas chromatograph equipped with a variable split. A glass capillary column (50 m × 0.25 mm i.d.) coated with 0.12 M Ni(II)-bis-(3-heptafluorobutyryl-1R, camphorate) in OV101 was used. The oven temperature was 70 °C with the flow of the nitrogen carrier gas at 0.4 ml/min. The split ratio was 1:50.

Total protein was determined according to Herbert et al. [23]. For dry weight measurements cells were centrifuged and washed twice in deionized water. Washed-cell suspensions (10–40 ml) were fixed with formaldehyde solution (40% v/v) and poured into dried pre-weighed pans. The weight of cells was determined after drying for 3 days at 70 °C.

RESULTS AND DISCUSSION

Characterisation of strain M90C

An ethene-utilising organism (designated M90C) was isolated in pure culture from soil using classical enrichment techniques. The bacterium is a Gram-positive coccus, characteristically occurring in pairs (diplococcus) and immotile in 'hanging-drop' preparations. On solid BMS medium, colonies appeared yellow, well separated, 1–2 mm in diameter, slightly raised and smooth with an entire edge. In liquid BMS medium, uniform turbidity, followed by a somewhat mucoidal deposit upon standing, was observed. Nutrient agar (with or without 5–10% w/v NaCl) supported growth; the colonies were yellow, sticky and produced slime. The biochemical properties of this strain are as follows: arginine and gelatin hydrolysis (+); esculin and starch hydrolysis (−); catalase, nitrate reduction and Kovac's oxidase (+); Simmons citrate, Voges Proskauer and urease (−).

On the basis of the above criteria, strain M90C was tentatively identified as a *Micrococcus* sp. [2].

Growth on various substrates

Unsaturated hydrocarbons (ethene, propene, 1-butene, butadiene) supported growth, whereas alkanes (methane, ethane, propane, butane) or *cis*-/*trans*-2-butenes did not, even after prolonged incubations (4 weeks). 1,2-Epoxyalkanes (C_2–C_4) were utilised after a long lag. Glucose, lactose, pyruvate and succinate (0.5% w/v), propionate, 1-propanol and 1,2-propanediol (0.2% v/v) also supported growth. The bacterium was able to grow well on higher concentrations of ethene (up to 50% v/v) despite its initial isolation at 5% (v/v), demonstrating its tolerance to the higher levels of olefin.

Substrate specificity

Ethene-grown *Micrococcus* sp. M90C accumulated epoxyalkanes (0.2–13 mM) from a wide range of olefins (Fig. 1 and Table 1) and was unable to hydroxylate alkanes or alkenes. This is consistent with the general finding that alkene-utilising bacteria only epoxidise olefins and do not hydroxylate alkanes or alkenes [19]. Control experiments with heat-killed cells indicated that the epoxides were produced enzymatically. Reaction mixtures lacking oxygen or substrate were also incapable of product formation.

The reactivity of a terminal double bond toward epoxidation was affected by the length of the alkyl chain. It was maximal for short-chain olefins (C_2–C_4), but decreased markedly as the chain was lengthened (C_8–C_{16}). *cis*- and *trans*-2-butenes were epoxidised at significantly lower rates than terminal gaseous olefins, whereas similar epoxidation activities were obtained with 1-octene and *trans*-2-butene. In peracid epoxidation reactions, no significant effect of carbon chain length on epoxidation of a terminal double bond is observed and even propene is as reactive as 1-octene. 2-Butenes are, however, 20-fold more reactive than terminal olefins, reflecting the stimulatory effect of alkyl substitution on electrophilic addition to the double bond [36]. *cis*-2-butene is expected to be more reactive than the *trans* isomer as its double bond is sterically less hindered and the methyl group is also rotated from planarity and tilted, resulting in a more strained structure than the *trans* isomer [1]. Microbial epoxidation of *trans*-2-butene was, however, 2-fold faster than the *cis* isomer (Table 1). This suggests that the *trans* configuration may be sterically favoured for oxidation.

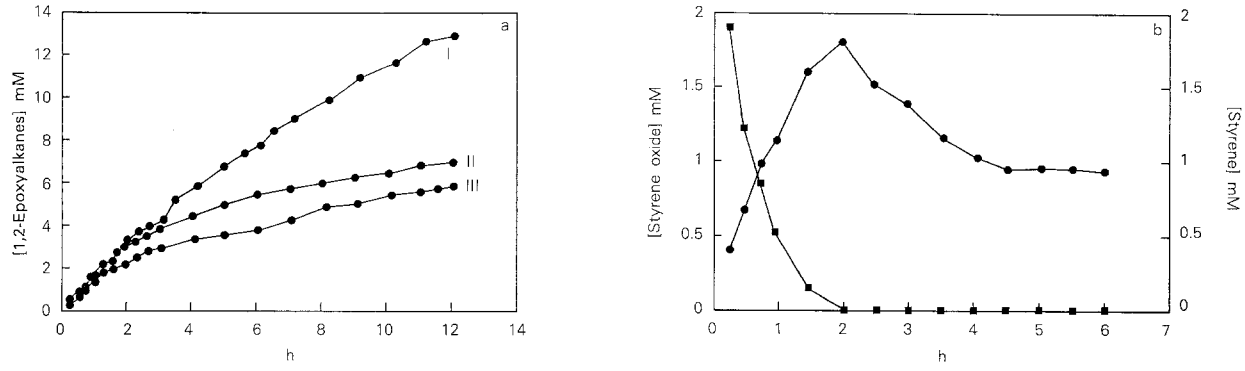

Fig. 1. Production of epoxides by *Micrococcus* sp. M90C. (a) I, 1,2-epoxybutane; II, epoxyethane; III, 1,2-epoxypropane. (b) ●, styrene oxide; ■, styrene. Reactions were carried out as stated in the legend to Table 1.

Micrococcus sp. M90C accumulated cyclohexene oxide and cyclopentene oxide (0.2 and 2 mM, respectively) from their corresponding olefins. Cyclohexene was oxidised at a similar rate to *cis*-2-butene, whereas a marked increase (19-fold) in activity was evident with cyclopentene (Table 1). This pattern of reactivity is also observed with peracids [1,36].

The organism accumulated styrene oxide and phenyl glycidyl ether (up to 2 mM) from their corresponding olefins (Fig. 1b). Styrene was epoxidised at a similar rate to propene and cyclopentene, whereas a 2-fold reduction in activity was observed with phenyl allyl ether (Table 1). In peracid epoxidation reactions, styrene is 17-fold less reactive than cyclopentene and 3-fold more reactive than propene, whereas phenyl allyl ether could be expected to be as reactive as propene [36].

Epoxyalkanes (C_2–C_{16}) were enzymatically hydrolysed, although at very slow rates which allowed them to accumulate. Further hydrolysis of the product was evident on prolonged incubations with the olefins (Fig. 1b). The epoxide-degrading enzyme(s) of *Micrococcus* sp. M90C also appears to have a broad substrate specificity. The slower product formation observed after 1–2 h of reaction may, therefore, be explained by the enzymatic hydrolysis of epoxyalkanes as well as product inhibition [20].

The results presented here clearly demonstrate that the substrate specificity of *Micrococcus* sp. M90C toward internal, terminal and aryl-substituted olefins is different

TABLE 1

Epoxidation of olefins by ethene-grown *Micrococcus* sp. M90C

Substrate	Accumulated product	Specific activity[a]	Specific productivity[b]
Ethene	epoxyethane	67	785
Propene	1,2-epoxypropane	48	563
1-Butene	1,2-epoxybutane	51	601
cis-2-butene	*cis*-2,3-epoxybutane	3	36
trans-2-butene	*trans*-2,3-epoxybutane	7	86
1-Octene	1,2-epoxyoctane	8	94
1-Hexadecene	1,2-epoxyhexadecane	0.05	0.6
Cyclopentene	cyclopentene oxide	37	438
Cyclohexene	cyclohexene oxide	2	25
Styrene	styrene oxide	38	445
Phenyl allyl ether	phenyl glycidyl ether	18	214

The bacterium was batch grown for 4 days with 5% (v/v) ethene. Reaction: 25 mM phosphate buffer, pH 7.0 at 30 °C. The bioconversion was started by injecting gaseous olefins (40% v/v) to displace an equal volume of air, whereas liquid olefins were added to a final concentration of 2 mM. Activities were determined during the first hour of reaction where epoxide formation was linear. Dry weight, 2.56 g/l (OD_{600}; culture, 0.48; resting cells, 15.0).

[a] nmol/min/mg protein.

[b] μmol/h/g dry weight.

from that expected on the basis of the chemical reactivity pattern in peracid epoxidation reactions. It should, however, be noted that these studies were carried out with intact cells and may, therefore, not truly reflect the reactions at the enzymatic level. Further conclusions as to the cause of the observed differences in the reactivity of various olefins can only be made on the basis of studies with a purified enzyme system(s).

Enantiomeric composition of epoxyalkanes

The enantiomeric composition of microbially produced epoxides (C_3–C_4) was investigated by chiral complexation gas chromatography [32]. To confirm that the two peaks observed with (±)1,2-epoxypropane corresponded to R- and S-enantiomers and not to any possible product formed by reaction of epoxypropane with the column material, the pure isomers were injected separately onto the column. Each isomer, however, produced only one peak. Comparison with known elution patterns [30,31,33] and peak area integration, showed that *Micrococcus* sp. M90C formed 1,2-epoxypropane (e.e. = 96%), R-1,2-epoxybutane (e.e. = 94%) and *trans*-(2R,3R)-epoxybutane (e.e. = 84%) from their corresponding olefins.

Stereoselectivity of epoxyalkane degradation

Biological resolution of racemic epoxyalkanes via enantioselective destruction of one stereoisomer is an alternative method of obtaining optically pure epoxides. Microsomal epoxide hydrolase has been extensively studied in recent years [30,34,40], but reports on the microbial enzyme(s) are scarce [35,39].

Metabolism of racemic 1,2-epoxypropane, 1,2-epoxybutane and *trans*-2,3-epoxybutane by ethene-grown *Micrococcus* sp. M90C was followed by head-space gas analysis on chiral complexation gas chromatography. Each isomer was quantified by plotting standard curves of peak areas against sample concentrations (methane as an internal standard). Both enantiomers of 1,2-epoxyalkanes (C_3–C_4) were simultaneously hydrolysed (Fig. 2a and b) to completion (9–10 h). A high enantiomeric excess of R-1,2-epoxyalkanes (e.e. = 94–96%), therefore, did not result from a stereoselective hydrolysis of the S-enantiomer, as evident by a constant R/S ratio during the reaction. Complete hydrolysis (24 h) of the (2S,3S)-enantiomer of (±)*trans*-2,3,epoxybutane, however, resulted in an enrichment in the (2R,3R)-enantiomer (Fig. 2c). Similarly, if epoxidation of *trans*-2-butene was non-stereospecific, a racemic production of the corresponding epoxide would be expected initially, followed by a rapid hydrolysis of one enantiomer to give the R configuration. The fact that this was not the case confirms that epoxidation of 1- and 2-olefins occurs stereospecifically.

Chemical synthesis of chiral *trans*-(2R,3R)-epoxybutane from (2S,3S)-tartaric acid involves eight different steps [31]. Kinetic resolution of racemic *trans*-2,3-epoxybutane by *Micrococcus* sp. M90C can, however, provide a simple and convenient one-step procedure for the preparation of the optically pure *trans*-(2R,3R)-enantiomer.

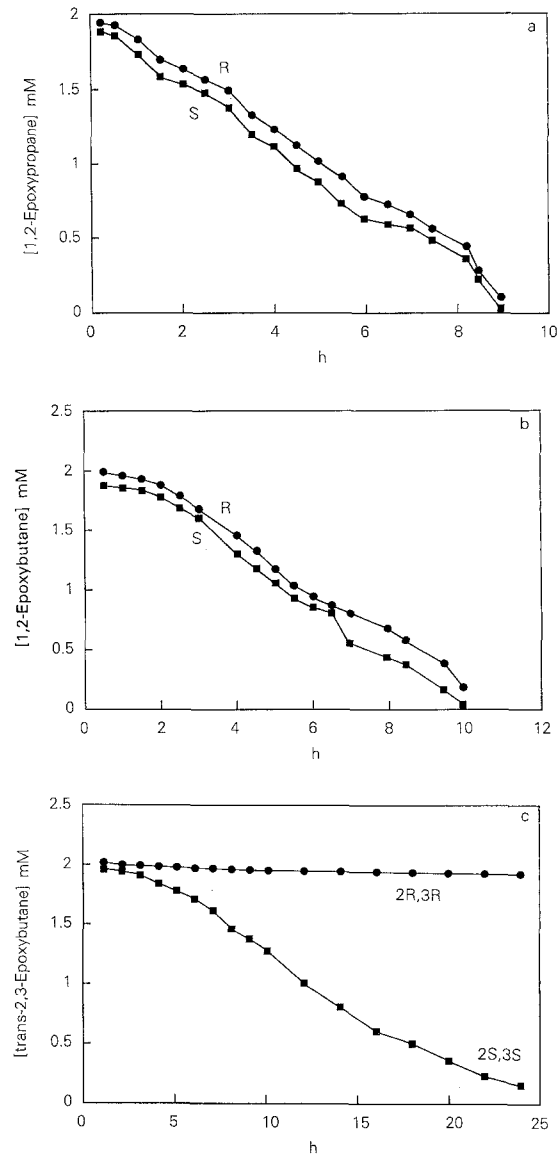

Fig. 2. Degradation of racemic epoxyalkanes by ethene-grown *Micrococcus* sp. M90C. (a) 1,2-Epoxypropane; (b) 1,2-epoxybutane; (c) *trans*-2,3-epoxybutane. Reaction conditions are as stated in the legend to Table 1. Each reaction was started by the addition of racemic epoxides to a final concentration of 2 mM.

The involvement of a specific monooxygenase

Cell-free extracts of ethene-grown *Micrococcus* sp. M90C were used to investigate the nature of the en-

TABLE 2

Production of epoxyethane by whole cells and cell-free extracts of *Micrococcus* sp. M90C

Fractions	Specific activity[a]	Relative activity (%)
Whole cells	60	100
(S) with NADH without BME	19	32
(S) with NADH and BME	0	0
(S) without NADH and BME	0	0

Reaction conditions are as stated in the legend to Table 1. The soluble fraction (S) was assayed in a total volume of 1 ml containing 5 μmol NADH. BME was added to a final concentration of 5 mM during the preparation of cell-free extracts.
[a] nmol/min/mg protein.

zyme involved in the epoxidation of ethene. The epoxidation activity was localised in the soluble fraction, which accumulated epoxyethane (1–2 mM) but was unable to hydroxylate alkanes or alkenes. This is in accordance with similar findings in whole cells. Formation of epoxyethane by the soluble fraction was dependent on the presence of both oxygen and NADH. No activity was observed in the absence of either or with heat-killed cells indicating the presence of a monooxygenase [5,7,12]. The cell-free ethene monooxygenase (EMO) catalysed the stereospecific formation of R-1,2-epoxypropane (e.e. = 95%), R-1,2-epoxybutane (e.e. = 92%) and *trans*-(2R,3R)-epoxybutane (e.e. = 83%) in the presence of NADH and oxygen. This is also consistent with the involvement of a monooxygenase in the production of chiral epoxides in whole cells. The activity was stable to short-term (7–10 days) storage at −40 °C in the absence of any stabilising agents but was completely inhibited in the presence of 5 mM β-mercaptoethanol (BME).

Alkene monooxygenases (AMO) reported to date suffer from low activity and instability [5,19]. Partial purification of the components of AMO from *Mycobacterium* spp. has been attempted. The enzyme complex was found to be unstable, suffering from a very low activity (0.25 nmol/min/mg protein in the crude extract) for the production of 1,2-epoxypropane [22]. Demonstration of a relatively stable cell-free activity in *Micrococcus* sp. M90C (Table 2) should make it an ideal system for future work on the enzymology of EMO.

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