Substrate range and enantioselectivity of epoxidation reactions mediated by the ethene-oxidising *Mycobacterium* strain NBB4

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Running Title: *Biocatalytic epoxidation by Mycobacterium strain NBB4*

Abstract

Mycobacterium strain NBB4 is an ethene-oxidizing micro-organism isolated from the estuarine sediments of Botany Bay. In pursuit of new systems for biocatalytic epoxidation, we report the capacity of strain NBB4 to convert a diverse range of alkene substrates to epoxides. A colorimetric assay based on 4-(4-nitrobenzyl)pyridine (NBP) has been developed to allow the rapid characterisation and quantification of biocatalytic epoxide synthesis. Using this assay we have demonstrated that ethene-grown NBB4 cells epoxidise a wide range of alkenes, including terminal (propene, 1-butene, 1-hexene, 1-octene and 1-decene), cyclic (cyclopentene, cyclohexene), aromatic (styrene, indene), and functionalised substrates (allyl alcohol, dihydropyran and isoprene). Apparent specific activities have been determined and range from 2.5 to 12.0 nmol min^{-1} per milligram of cell protein. The enantioselectivity of epoxidation by *Mycobacterium* strain NBB4 has been established using styrene as a test substrate; (*R*)-styrene oxide is produced in enantiomeric excesses greater than 95%. Thus the ethene monooxygenase of *Mycobacterium* NBB4 has a broad substrate range and promising enantioselectivity, confirming its potential as a biocatalyst for alkene epoxidation.

Key Words

biocatalysis; epoxidation; alkene; Mycobacterium; soluble di-iron monooxygenase; non-heme iron; oxidation

Introduction

Epoxides are widely used as intermediates in the synthesis of complex molecules (Breuer et al. 2004; Izawa and Onishi 2006; Oyama 2008). They are most commonly made from alkenes via the Prilezhaev reaction (peracid epoxidation) (Hagen 2007), Sharpless epoxidation (Pfenninger 1986) or Jacobsen epoxidation (McGarrigle and Gilheany 2005). However there are significant limitations with standard approaches to epoxide synthesis, in particular safety and environmental concerns associated with the use of peroxides and peracids, chlorinated solvents and transition metal catalysts (Constable et al. 2007). A recent survey of the pharmaceutical industry by the ACS Green Chemistry Institute identified epoxidation as equal third on a list of reactions for which “better reagents” are required (Constable et al. 2007).

There is considerable interest in biocatalytic approaches using either whole cells or enzyme preparations for epoxide synthesis (Nolan and O'Connor 2008; Archelas and Furstoss 1999; Meyer and Turner 2009). Biocatalytic reactions occur under mild aqueous conditions, are amenable to scale-up, and the enzyme catalysts are renewable, non-toxic and biodegradable (Meyer and Turner 2009). Other key properties of biocatalysts (eg substrate specificity, specific activity, enantioselectivity, stability and solvent tolerance) can be improved using recombinant DNA techniques via rational mutagenesis and directed evolution (Reetz 2009).

Monooxygenases (MOs) catalyse the addition of one oxygen atom from molecular oxygen into their substrate and thus can produce epoxides from alkenes (Harayama et al. 1992). There are several biochemically distinct classes of MOs, and their potential utility as biocatalysts varies (Harayama et al. 1992). Activation of molecular oxygen is achieved via iron, copper, flavin or pteridine-based cofactors in the different MO classes, but it is generally the fine detail of active site structure rather than specific co-factor chemistry that controls the substrate range, stereo- and regioselectivity of MO catalysis. MOs have a range of physiological roles, spanning primary (growth on various hydrocarbon substrates) and secondary metabolism (including secondary metabolite synthesis and detoxification of xenobiotics). The role of the MO indirectly influences its potential utility as a biocatalyst, and the catalytic properties of a given MO reflect the evolutionary pressures behind its physiological role. Enzymes needed for primary metabolism are more likely to be expressed at high levels, which is especially relevant for whole cell biocatalysis applications.

Aerobic bacteria that grow on alkenes, alkanes and aromatics use MOs to oxidize hydrocarbon substrates to epoxides or alcohols (Leak et al. 2009; Fruetel et al. 1992; Chang et al. 2004; Wubbolts et al. 1994; Gallagher et al. 1997). These bacterial MOs have broad substrate ranges and some are very stereoselective, producing epoxides in high enantiomeric excess (Gallagher et al. 1997; Owens et al. 2009; Mahmoudian and Michael 1992). The MOs of bacteria that grow on alkene substrates are of particular interest for biocatalytic epoxide synthesis. The best-characterized alkene MOs are those from propene-degrading organisms, and these enzymes have broad substrate range and high stereoselectivity (Takagi et al. 1990). Ethene MOs are less well-investigated, but initial studies suggest that these enzymes are also able to produce chiral epoxides in very high enantiomeric excesses (>98%) (Owens et al. 2009).

Although the MOs of alkene-utilising organisms have favorable properties for application to biocatalysis, there is the potential drawback that the epoxide products can be further metabolized. In the natural cell system the epoxides produced from ethene **1** and related substrates are detoxified and routed towards central metabolism by the epoxyalkane:CoM transferase enzyme (EtnE or XecA), which is unique in aerobic bacteria in its requirement for coenzyme M (CoM, mercaptoethanesulfonate) (Krishnakumar et al. 2008). In the ethene assimilation pathway, ethene **1** is oxidised to ethene oxide **2**, which is conjugated to CoM to give hydroxyethyl-CoM **3**, and proceeds into central metabolism by a pathway proposed to require the addition of coenzyme A and carboxylation to ultimately yield malonate semialdehyde (Figure 1) (Mattes et al. 2010). Thus the yield of epoxides from whole cell biocatalysis by alkene-degrading organisms is influenced by properties of both the MO and the downstream epoxyalkane:CoM transferase. We postulate that organisms evolved to use the smaller substrate ethene will give better epoxide yields with larger substrates than organisms optimized for growth on propene, providing the EtnE is less catholic than the ethene MO.

<Figure 1>

In a previous study, we isolated the ethene-oxidizing *Mycobacterium* strain NBB4 by enrichment on ethene **1** as sole carbon source from an inoculum of estuarine sediment, and demonstrated that this bacterium contains multiple MO genes of

potential interest for biocatalysis (Coleman et al. 2006). More recently, we have recovered complete gene clusters encoding the various MO enzymes of NBB4 (Genbank GU174750-GU174754) and used proteomics methods to confirm this ethene MO is the dominant cell monooxygenase expressed during growth on ethene as sole carbon source (Coleman *et al.*, submitted). The aims of the current study were to develop a rapid method for monitoring and quantifying the biocatalytic production of epoxides, to use this method to investigate the substrate scope of ethene-grown *Mycobacterium* NBB4 cells, and to determine the kinetic parameters and enantioselectivity of epoxidation by *Mycobacterium* NBB4.

Materials and Methods

Growth of bacteria.

Mycobacterium strain NBB4 (Coleman et al. 2006) was grown aerobically at 30 °C in MSM minimal medium (Coleman et al. 2002) containing 0.02% (v/v) Tween 80. Cultures (300 mL) were grown in gas-tight flasks (1000 mL), with ethene added as the carbon and energy source (10% of headspace volume), with shaking at 200 rpm. Cultures were started from an optical density (OD_{600}) of 0.05 and grown to mid-log phase (OD_{600} 0.4-0.7). The cells were harvested by centrifugation (3100 *g*, 10 min, 4 ˚C), and washed twice with phosphate buffer (20 mM K_2HPO_4, pH 7.0, containing 0.02% (v/v) Tween 80).

Mycobacterium strain NBB4 has been deposited in the Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan, http://www.jcm.riken.jp/ under accession number JCM 17769.

NBP assay

A 2 mL glass vial containing 4-(4-nitrobenzyl)pyridine (NBP) solution (500 µL, 100 mmol L^{-1}) in ethylene glycol was placed inside a 16 mL bottle containing ethene-grown NBB4 cells (4 mL) at an OD_{600} of 13.0 ± 0.5 (a protein concentration of 0.86 mg mL^{-1}). The outer bottle was crimp-sealed with a Teflon-faced butyl rubber stopper, while the inner vial was left open. Alkene substrates were added either as gas in the headspace (10% v/v), or liquid to the outer vial (30-80 µmol), and the bottles incubated at 30 ˚C with shaking at 200 rpm for 24 h. Triethylamine solution (0.5 mL, 1:1 v/v in acetone) was added to the inner vial and the absorbance at 550 nm was measured immediately. Two negative controls were included in all experiments: one

with heat-killed cells (autoclaved 121 °C, 15 min), and one with no alkene added. None of these controls gave positive results in any of the NBP assays performed, except for the methyl vinyl ketone:killed cells assay (discussed further below).

Determination of molar absorptivities

Stock solutions of propene oxide, 1-butene oxide, styrene oxide, cyclopentene oxide and cyclohexene oxide in acetone were prepared (10-100 mmol L^{-1}). To calculate the molar absorptivity coefficient ε, epoxide solution (50 μL) was mixed with NBP (450 μL, 100 mmol L^{-1} in ethylene glycol), then triethylamine solution (500 μL, 1:1 v/v in acetone) was added immediately and the absorbance at 550 nm (A_{550}) measured. The ε value was then calculated according to the Beer-Lambert Law $A = \varepsilon cL$ (where $A = A_{550}$, c = epoxide concentration in mmol L^{-1}, and L = path length (1 cm in these assays)). The apparent molar absorptivity coefficient ε^* was calculated similarly, except that epoxide solutions (50 μL) were added to phosphate buffer (4 mL) in the outer vial of a 2-vial system, and the NBP reagent to the inner vial, then incubated 24 h with shaking before triethylamine was added to the inner vial and the absorbance measured.

Kinetics of 1-butene oxidation

1-Butene (15% of headspace volume) was added to crimp-sealed vials (16 mL) containing either live or heat-killed ethene-grown NBB4 cells in phosphate buffer (4 mL), which were incubated with shaking as above. Headspace samples (100 μL) were analysed at intervals by GC-FID (HP 5890-IIE, HP PLOT-Q column); 1-butene and 1-butene oxide were identified based on their retention times relative to authentic standards, and quantified using external standards prepared under identical conditions (4 mL phosphate buffer in 16 mL vial at 30 °C).

Production of styrene oxide

One-Phase System

Ethene-grown NBB4 cells in phosphate buffer (500 μL, OD_{600} = 14 ± 3; 0.92 mg protein mL^{-1}) were added to a 2 mL crimp-seal bottle and neat styrene was added to achieve a final concentration of 10 mmol L^{-1}. The reaction was incubated with shaking for 24 h. Diethyl ether (100 μL) was added and the resulting suspension centrifuged (16,000 *g*, 2 min) to precipitate cellular materials. The ether layer was dried over sodium sulfate, and analysed by chiral GC.

Two-Phase System

Ethene-grown NBB4 cells in phosphate buffer (400 µL, OD_{600} = 14 ± 3; 0.92 mg protein mL^{-1}) were mixed with styrene (100 µL) in a 2 mL crimp seal bottle, incubated as above for 24 h, then the styrene phase was removed, dried, and analysed directly by chiral GC. Controls using heat-killed cells and live cells with no styrene were run under the same conditions.

Chiral GC and LC analyses

To obtain authentic samples of each enantiomer a solution of racemic styrene oxide (1 mL, 200 $mmolL^{-1}$, in 98:2 hexane:isopropanol) was separated using chiral HPLC (Waters 510, ChiralPak AD-H column, 98:2 v/v hexane:isopropanol mobile phase; retention times 10.1 min and 11.2 min). The fractions were concentrated under reduced pressure and optical rotation measured on a Perkin-Elmer Model 341 Polarimeter at 20 ºC with sodium lamp: first peak $[\alpha]_{589}^{20}$ = +9.9 (*c* = 0.004 in hexane/ isopropanol, *l* = 0.5); second peak $[\alpha]_{589}^{20}$ = –6.7 (*c* = 0.003 in hexane/ isopropanol, *l* = 0.5). An authentic sample of (*R*)-(+)-styrene oxide (Sigma-Aldrich) analysed under the same conditions returned $[\alpha]_{589}^{20}$ = +10.6 (*c* = 0.004 in hexane/ isopropanol, *l* = 0.5). Furthermore, the literature reports GC retention times on a Cyclodex-B column of 14.1 min for (*R*)-(+)-styrene oxide and 14.5 min for (*S*)-(-)-styrene oxide (Nickerson et al. 1997). Based on this combined analysis, the first peak isolated from the HPLC separation was determined to be (*R*)-(+)-styrene oxide and the second (*S*)-(-)-styrene oxide.

Chiral GC analysis using a HP 5890A chromatograph with FID detector and J&W Cyclodex-B column gave retention times for the two enantiomers of 13.6 min for the (*R*)-enantiomer and 14.0 min for the (*S*)-enantiomer. Analysing the styrene oxide produced by *Mycobacterium* NBB4 in the same way revealed that this micro-organism preferentially produces (*R*)-styrene oxide from styrene.

Results

Development of two-vial colorimetric assay for epoxide detection

The reagent 4-(4-nitrobenzyl)pyridine (NBP, **4**) is used for the detection and quantification of various alkylating agents including epoxides (Epstein et al. 1955; Kim and Thomas 1992; Coleman and Spain 2003). The pyridine nitrogen of NBP opens the epoxide by nucleophilic attack (Figure 2), and in the presence of base, a purple chromophore develops. The NBP reaction offers advantages for epoxide

detection compared to gas chromatography including simplicity, speed, sensitivity, applicability to diverse epoxides, and adaptability to high-throughput screening.

<Figure 2>

For the present study, a two-vial ('vial-in-vial') variant of the NBP assay (McClay et al. 2000) was adapted and used to test biocatalytic epoxide production by *Mycobacterium* NBB4. The outer of the two vials is crimp-sealed and contains cells in aqueous buffer; the inner vial is uncapped and contains NBP in ethylene glycol. Alkenes are provided either via the gas phase or into the outer aqueous phase. This differs from a standard end-point assay because epoxides can diffuse continuously from the outer aqueous reaction mixture through the headspace into the inner vial, where the epoxide-NBP conjugate accumulates. Preliminary experiments using *Mycobacterium* NBB4 cells indicated that the 2-vial approach gave greater sensitivity compared to an end-point assay using the same reagents. This could be due to minimization of epoxide metabolism by the cells, minimization of aqueous hydrolysis, and reduction of toxic effects of the epoxide on cells.

To be made quantitative, the NBP assay must be calibrated to the molar extinction coefficients of each NBP-epoxide conjugate. In a two-vial method, the rate of epoxide transfer between the outer and inner vials must also be accounted for.

Molar extinction coefficients (ε) of five representative NBP-epoxide conjugates were determined by direct addition of epoxide to NBP (Table 1), and are in broad agreement with values previously reported (Agarwal et al. 1979). The molar extinction coefficients measured for the terminal epoxide-NBP conjugates (propene, 1-butene, and styrene oxides) are 5- to 10-fold higher than values for the cyclic epoxide-NBP conjugates (cyclopentene and cyclohexene oxides), presumably because the cyclic epoxides are less reactive towards NBP due to steric or electronic factors.

Apparent extinction coefficients (ε^*) were then determined for indirect addition of epoxide to NBP by diffusion in the two-vial system. The percentage transfer of epoxide from the outer vial and headspace into the inner vial was calculated as $\varepsilon^*/\varepsilon$, and ranged from 23 to 48%, indicating that factors including diffusion, gas/liquid-partitioning and aqueous hydrolysis limit the maximum possible recovery of epoxides in the two-vial NBP assay.

<Table 1>

Substrate scope of Mycobacterium NBB4 epoxidation

Initial experiments were performed to test the effect of growth medium on epoxidation, using 1-butene and styrene as test substrates. NBB4 cells grown in standard media (acetate as carbon source) did not epoxidise either alkene. In contrast, cells grown on ethene did produce epoxides, implicating the ethene-inducible MO EtnABCD as the enzyme responsible for this activity. Ethene-grown whole cells of wild-type *Mycobacterium* NBB4 were used for all subsequent biotransformations, due to the inducible nature of the ethene MO, its instability in cell extracts, and difficulties with overexpressing this enzyme in heterologous hosts (Mattes et al. 2010; Hartmans et al. 1991).

A wide range of alkene substrates were screened using the vial-in-vial NBP assay (Table 2). Ethene-grown NBB4 cells produced epoxides from an array of terminal (**7**–**9**, **12**–**14**), cyclic (**10**, **11**, **16**, **18**) and functionalised (**15**, **16**, **17**) alkenes. Of the 14 alkenes tested, only cyclooctene **19** and limonene **20** failed to give detectable yields of epoxide products. Epoxidation of propene, 1-butene, styrene, cyclopentene and cyclohexene was further characterised and quantified to determine the *percentage conversion*, *apparent specific activity* and *volumetric efficiency* for turnover of these representative substrates by *Mycobacterium* NBB4 (Table 3).

<Table 2; Table 3>

Analysis of 1-butene oxidation and 1-butene oxide accumulation

To characterise the efficiency of *Mycobacterium* NBB4 as a catalyst for the epoxidation of alkenes other than its natural substrate ethene, turnover of 1-butene **8** to 1-butene oxide by NBB4 was followed by GC analysis (Figure 3). Specific activities of 24.0 ± 2.0 nmol min^{-1} per milligram of protein for 1-butene oxidation and 4.4 ± 0.5 nmol min^{-1} mg^{-1} for 1-butene oxide accumulation were calculated.

<Figure 3>

Stereoselectivity

To investigate the enantioselectivity of epoxide production in strain NBB4, two different reaction conditions were studied using styrene **9** as a test substrate: (i) an aqueous solution of styrene (10 mmol L^{-1}); and (ii) a biphasic system with styrene as the organic phase. The latter system is designed so that the styrene oxide can partition into the organic phase, minimising cellular toxicity and further metabolism, while also removing the need for a separate product extraction step (Sardessai and

Bhosle 2004; Heipieper et al. 2007). A potential problem with this approach is that styrene itself can be toxic to cells at higher concentrations (Leon et al. 1998).

In the single-phase experiments using homogenous aqueous solutions, volumetric efficiencies of 2.8 ± 0.2 mg L^{-1} h^{-1} were calculated based on ether-extraction of the culture supernatant after 24 hours. In the two-phase system, an efficiency of 5.2 ± 0.4 mg L^{-1} h^{-1} was calculated from direct GC analysis of the styrene phase. Ether extraction of aqueous assay mixtures yielded styrene oxide in high enantiomeric excess (92-98%) while the enantioselectivity of the biphasic reaction was lower (66-86%). Chiral GC analysis and comparison with authentic samples of (*S*) and (*R*)-styrene oxide show that NBB4 preferentially produces the (*R*)-enantiomer (Figure 4).
<Figure 4>

Discussion

Development of two-vial colorimetric assay for epoxide detection

The two-vial NBP assay developed to screen biocatalytic epoxide production by *Mycobacterium* NBB4 allows sensitive, quantitative, and rapid general-purpose screening of biocatalytic epoxidation. The assay is contingent on at least some of the epoxide escaping cellular metabolism, it being sufficiently volatile to diffuse into the ethylene glycol phase via the headspace, and it then reacting with NBP. This method is not suitable for the small subset of alkenes that react directly with NBP, and requires that the molar extinction coefficient of the NBP-epoxide conjugate be known, readily estimated, or determined experimentally.

Substrate scope of Mycobacterium NBB4 epoxidation

High levels of epoxide formation were seen with 1-butene **8**, cyclopentene **10** and 1-octene **12**, moderate turnover with propene **7**, styrene **9**, cyclohexene **11** and 1-hexene **13**, and low conversion for 1-decene **14**, isoprene **15**, dihydropyran **16**, allyl alcohol **17** and indene **18**; cyclooctene **19** and limonene **20** gave no detectable epoxide product (Table 2). The largest substrates tested (1-decene, cyclooctene, limonene) gave the poorest conversion, but otherwise there is no obvious relationship between the size or shape of the test substrates and the level of epoxide generation. Estimated epoxide yields are also influenced by differences in the volatility of the product epoxides (which must diffuse into the NBP-containing phase to be detected) and the relative stability of these epoxides to the biocatalysis conditions, in particular to hydrolytic enzymes such as epoxide hydratases (Weijers

and de Bont 1999); the breakdown of indene oxide for example is particularly facile (Weijers 1997).

The failure of the NBP assay to detect oxidation of *cis*-cyclooctene **19** and limonene **20** is most likely because these alkenes are not turned over by the ethene MO. However this observation could also be due to another of the multiple variables intrinsic to the assay: it is possible that these alkenes are oxidised but the resulting epoxides are metabolised or broken down abiotically with particular rapidity, or that there is particularly inefficient transfer of these epoxides into the ethylene glycol phase. It is also possible (though unlikely) that these epoxides do not react with NBP.

The demonstration that NBB4 does epoxidise the functionalized substrates 3,4-dihydropyran **16** and allyl alcohol **17** expands the potential utility of this biocatalyst. Although bacterial epoxidation of 3-hydroxypentadiene (Zaks and Dodds 1995) and allyl ethers (Fu et al. 1991) has been reported, to the best of our knowledge this is the first report of bacterial epoxidation of allyl alcohol, which represents an enzymatic corollary to the Sharpless epoxidation (Pfenninger 1986). Both functionalised alkenes gave strong NBP reactions when incubated with NBB4 cells, but the purple chromophore was unstable and decomposed quickly (within minutes). Thus it is likely that estimates of epoxide formation from functionalised alkenes under-represent the true level of turnover. *Mycobacterium* NBB4 cells also turned over methyl vinyl ketone (MVK, data not shown), however the direct reactivity of MVK itself with NBP (via Michael addition) complicates analysis.

Oxy-functionalised alkenes are expected to be poorer substrates for biocatalytic epoxidation than similarly-sized terminal aliphatic or cyclic alkenes due to their more polar nature. All of the soluble di-iron MO enzymes characterised to date (including ethene MO from NBB4) are believed to contain a channel of hydrophobic amino acids leading to the active site. These hydrophobic residues have been implicated in substrate recognition, binding and regiospecificity (Leahy et al. 2003; Notomista et al. 2009; Pikus et al. 1997) and may hinder the access of polar compounds such as allyl alcohol and dihydropyran to the active site.

There are three potential epoxide products from the turnover of isoprene **15**, resulting from reaction at just the 1-alkene, just the 3-alkene, or at both positions. We propose that the observed product results from a single epoxidation at the mono-substituted 3-alkene, on the basis that *Mycobacterium* NBB4 readily epoxidises a range of

monosubstituted alkenes (Table 2) but fails to react with either the gem-di-substituted or tri-substituted alkenes of limonene **20**.

Analysis of 1-butene oxidation and 1-butene oxide accumulation

Non-stoichiometric accumulation of 1-butene oxide was observed (only 25% after 5 hours), and both the kinetics and yield of this reaction show that 1-butene oxide is rapidly transformed, by either abiotic or bacterial reactions. To test the rate of epoxide breakdown, cell suspensions were given 1-butene oxide as a substrate, and tested using the NBP assay after 24 hours against a control using heat-killed cells. No epoxide was detectable in the live-cell sample, but 75% of the epoxide remained in the heat-killed control. We propose that 1-butene oxide is metabolised by the EtnE enzyme of NBB4, resulting in the low apparent rate and yield of epoxide accumulation in cell suspensions. Bromoethanesulfonate is a specific inhibitor of propene oxide metabolism (Boyd et al. 2006). This was investigated as a means of inhibiting 1-butene oxide metabolism in NBB4, but did not afford any greater accumulation of the desired epoxide.

The specific activity observed for 1-butene oxidation (24.0 ± 2.0 nmol min^{-1} mg^{-1}) compares well to rates for 1-butene epoxidation by related systems (Mahmoudian and Michael 1992). Of seven ethene- and propene-utilising bacteria characterised by Mahmoudian and Michael, six show specific activities for 1-butene epoxidation in the range 5–26 nmol min^{-1} mg^{-1}. These include *Micrococcus, Staphylococcus* and *Aerococcus* species. Only *Micrococcus* sp. M90C, at 51 nmol min^{-1} mg^{-1}, displayed a discernibly higher rate of butene turnover than *Mycobacterium* NBB4.

The efficient turnover of both 1-butene and 1-butene oxide suggest that NBB4 might grow on 1-butene; this was tested and growth was observed, but at a slower rate than growth on ethene.

Stereoselectivity

The stereoselectivity of ethene MO enzymes has not been well-investigated, but data from *Nocardioides* JS614 and other alkene-utilising bacteria indicate a capacity for highly stereoselective reactions with C_3-C_4 substrates (Table 4).

Based on the volumetric efficiency data obtained, it is evident that *Mycobacterium* strain NBB4 cells epoxidise styrene better in the presence of the styrene phase. Notably the yields of epoxide obtained show no evidence of styrene toxicity. We also noted that NBB4 performs well in a variant of the NBP assay using acetone instead

of ethylene glycol (data not shown), in which cells are subjected to high concentrations of acetone via the headspace. Organic solvent tolerance is a very beneficial trait for an industrial biocatalyst (Heipieper et al. 2007; Leon et al. 1998). The efficiency of styrene oxide production estimated from these GC assays is lower than that calculated from NBP assay (18.0 ± 5.8 mg L^{-1} h^{-1}, Table 3), indicating that the ethylene glycol-NBP phase in the 2-vial system is more effective than either a styrene phase or an aqueous phase for styrene oxide accumulation.

The observation of higher enantioselectivity in single-phase experiments (*ee* = 92-98%) than under biphasic conditions (*ee* = 66-86%) may occur because high styrene concentrations induce expression of a second, less stereoselective MO that also contributes to styrene oxide production, although this hypothesis was not investigated further.

The preferential formation of (R)-styrene oxide observed using *Mycobacterium* strain NBB4 is consistent with data for other epoxidations using ethene and propene MOs (Rigby et al. 1994; Nickerson et al. 1997; Owens et al. 2009). The average optical purity of (*R*)-styrene oxide obtained using NBB4 cells in the single phase experiments is 95%, which compares well with enantiomeric excesses reported previously for biocatalytic epoxidation (Table 4).

<Table 4>

Potential of Mycobacterium NBB4 for biocatalytic epoxidation

Optimisation of the NBP assay has enabled a rapid quantitative investigation of the oxidative capacity of the ethene MO in *Mycobacterium* NBB4, and the definition of some limits to the scope of this biocatalyst. Ethene-grown NBB4 cells produced epoxides from terminal aliphatic alkenes, aromatic alkenes and functionalised alkenes ranging in size from C_2-C_{10}. To the best of our knowledge, the epoxidation of dihydropyran **16** and allyl alcohol **17** using bacterial systems has not been previously reported. The ethene MO of NBB4 exhibits little preference for terminal vs cyclic alkenes, and high yields of epoxide were obtained with C_4-C_8 terminal alkenes and C_5-C_6 cycloalkenes. Activity decreases quickly for substrates larger than C_8 (linear) or C_6 (cyclic) although epoxidation is still observed for indene (C_9) and 1-decene (C_{10}), and the broad substrate range of the ethene MO confirms that *Mycobacterium* NBB4 has considerable promise as a generic tool for the epoxidation of alkenes in organic synthesis. Site-directed mutagenesis and directed evolution experiments will

afford a better understanding of the roles of specific residues in ethene MO activity and stereoselectivity, and bring modified catalysts with enhanced activity for specific alkene substrates. Genome-sequencing of strain NBB4 is in progress (U.S. Joint Genome Institute Community Sequencing Program CSP-215), and will greatly assist further work in the use of this bacterium for biocatalysis.

Whole-cell biocatalysis has advantages in terms of cost, stability and cofactor requirements (Duetz et al. 2001) but can be limited because other metabolic reactions consume the desired product. In the present study, *Mycobacterium* NBB4 cells consumed much of the 1-butene oxide that was produced from 1-butene. We propose two ways to solve this problem and increase epoxide yields: molecular genetic strategies including heterologous expression of the MO genes (Chan Kwo Chion et al. 2005) and knockout of the epoxide-metabolic genes (Han et al. 2006); and the development of improved biphasic solvent conditions, to isolate epoxide products from metabolism while also minimising abiotic hydrolysis and epoxide reactions with cellular macromolecules (Leon et al. 1998; Koskinen and Plná 2000).

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Tables

Table 1 Molar extinction coefficients of NBP-epoxide conjugates and quantification of epoxide transfer in the two-vial assay

Epoxide	ε (L mol^{-1} cm^{-1}) [a,b]	ε* (L mol^{-1} cm^{-1}) [b,c]	Epoxide transfer (%) [d]	Sensitivity limit (μM) [e]
Propene oxide	8900 ± 100	2080 ± 70	23	10 (95)
1-Butene oxide	6700 ± 400	2480 ± 90	37	20 (50)
Styrene oxide	6600 ± 200	3200 ± 200	48	20 (50)
Cyclopentene oxide	205 ± 2	61 ± 4	30	1000 (2000)
Cyclohexene oxide	950 ± 40	340 ± 20	36	200 (500)

[a] Extinction coefficient (ε) determined by adding the epoxide directly to NBP **4**.

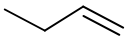
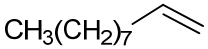
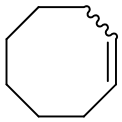
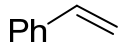
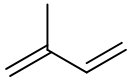
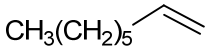
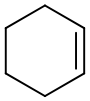
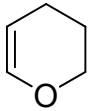
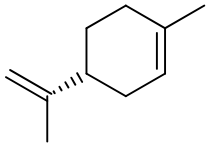
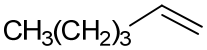
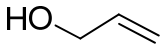
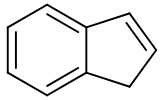
[b] Values shown are the average of 3 independent experiments; errors represent the standard error of the mean.

[c] Apparent extinction coefficient (ε*) measured by adding epoxide to phosphate buffer in the outer vial of a two-vial assay, NBP reagent in the inner vial.

[d] Transfer of epoxide from outer vial to inner vial, shown as the percentage of epoxide recovered in the inner vial after 24 h (calculated as ε*/ε).

[e] Sensitivity limits for the direct epoxide-NBP addition and (indirect addition of epoxide via the two-vial system). The sensitivity limit is defined as the amount of epoxide required to give an absorbance reading ≥ 0.10.

Table 2 Substrate scope of alkene epoxidation by ethene-grown cells of *Mycobacterium* NBB4[a,b]

High Turnover [c]	Moderate Turnover [d]	Low Turnover [e]	No Turnover
 1-Butene **8**	 Propene **7**	 1-Decene **14**	 Cyclooctene **19**
 Cyclopentene **10**	 Styrene **9**	 Isoprene **15**	
 1-Octene **12**	 Cyclohexene **11**	 Dihydropyran **16**	 Limonene **20**
	 1-Hexene **13**	 Allyl alcohol **17**	
		 Indene **18**	

[a] Turnover levels estimated using apparent extinction coefficients (ε*) detailed in Table 1. For substrates not listed in Table 1, ε* was estimated as follows: for other terminal alkenes (1-hexene, 1-octene, 1-decene, isoprene, and allyl alcohol) the average of ε^* values for propene, 1-butene, and styrene oxides (2600 M^{-1} cm^{-1}) was used; for 3,4-dihydropyran oxide, the ε^* value for cyclohexene oxide was used, for indene oxide, the ε^* value for cyclopentene oxide was used.

[b] 3 independent experiments performed with each substrate, except 1-hexene and indene (average of 2 experiments) and isoprene (single experiment).

[c] A_{550} indicates yield >70% using the vial-in-vial assay and apparent extinction coefficient.

[d] A_{550} indicates yield 25-70% using the vial-in-vial assay and apparent extinction coefficient.

[e] A_{550} indicates yield <25% using the vial-in-vial assay and apparent extinction coefficient.

Table 3 Quantification of alkene epoxidation by ethene-grown cells of *Mycobacterium* NBB4

Substrate	Amount of Epoxide (μmol) [a,b]	Conversion (%) [a,c]	Apparent specific activity [a,d]	Volumetric efficiency [a,e]
Propene **7**	12.3 ± 0.4	25 ± 1	2.6 ± 0.1	9.1 ± 0.3
1-Butene **8**	34 ± 2	70 ± 4	7.1 ± 0.1	31 ± 0.4
Styrene **9**	15 ± 3	36 ± 7	2.5 ± 0.8	18 ± 5.8
Cyclopentene **10**	53 ± 4	81 ± 12	12.0 ± 2.0	61 ± 10
Cyclohexene **11**	20 ± 2	39 ± 4	4.2 ± 0.7	25 ± 4.1

[a] Values are the average of 3 independent experiments, errors represent the standard error of the mean.

[b] Amount of epoxide produced (μmol) in 24 hours.

[c] Amount of epoxide produced as mole percent of amount alkene used.

[d] Specific activity as nmol epoxide produced per minute per milligram of protein (assuming a linear reaction rate over the time period).

[e] Volumetric efficiency calculated as mg L^{-1} h^{-1} epoxide product.

Table 4: Stereoselectivity of MO-mediated epoxidations

Enzyme	Product	ee (%)	Organism	Reference
Ethene MO	styrene-7,8-oxide	95 (*R*)	*Mycobacterium* NBB4	This study
Ethene MO	propene oxide	98 (*R*)	*Nocardioides* JS614	(Owens et al. 2009)
Propene MO	propene oxide	83 (*R*)	*Gordonia* B-276	(Takagi et al. 1990)
Propene MO	styrene-7,8-oxide	69 (*R*)	*Gordonia* B-276	(Takagi et al. 1990)
Propene MO	styrene-7,8-oxide	93 (*R*)	*Mycobacterium* M156	(Rigby et al. 1994)
Isoprene MO	styrene-7,8-oxide	87 (*R*)	*Rhodococcus* AD45	(Vlieg et al. 2000)
Styrene MO	styrene-7,8-oxide	> 99 (*S*)	*Pseudomonas* VLB120	(Panke et al. 1998)
Styrene MO	styrene-7,8-oxide	> 99 (*S*)	*Pseudomonas* SN1	(Bae et al. 2006)
Xylene MO	styrene-7,8-oxide	92 (*S*)	*Pseudomonas putida*	(Wubbolts et al. 1994)

Figure Legends

Fig. 1 Initial reactions of ethene metabolism in *Mycobacterium* spp. (modified from (Mattes et al. 2010))

Fig. 2 Reaction of epoxides with 4-(4-nitrobenzyl)-pyridine (NBP) and triethylamine that is the basis of the colorimetric assay

Fig. 3 Oxidation of 1-butene to 1-butene oxide by NBB4 cells (○ = butene oxide, live cells; □ = butene, live cells; △ = butene, killed-cell control; ◇ = butene oxide, killed-cell control)

Fig. 4 Epoxidation of styrene **9** can give rise to two enantiomeric epoxides, **21** and **22**. Mycobacterium NBB4 forms the (*R*) epoxide **21** in >95% enantiomeric excess