Biological conversion of gaseous alkenes to liquid chemicals

Shuchi H. Desai^{1,2}, Irina Koryakina^{1,2,3}, Anna E. Case¹, Michael D. Toney^{1,*}, Shota Atsumi^{1,*}

¹Department of Chemistry, University of California–Davis, Davis, California, USA.

²These authors contributed equally to this work.

^{*}To whom correspondence may be addressed: Email: satsumi@ucdavis.edu and mdtoney@ucdavis.edu

³Present address: Intrexon Corporation, South San Francisco, California, USA.

Keywords: Biological gas to liquid conversion, Metabolic engineering, Alkenes, Diols

Abstract

Industrial gas-to-liquid (GTL) technologies are well developed. They generally employ syngas, require complex infrastructure, and need high capital investment to be economically viable. Alternatively, biological conversion has the potential to be more efficient, and easily deployed to remote areas on relatively small scales for the utilization of otherwise stranded resources. The present study demonstrates a novel biological GTL process in which engineered *Escherichia coli* converts C2-C4 gaseous alkenes into liquid diols. Diols are versatile industrially important chemicals, used routinely as antifreeze agents, polymer precursors amongst many other applications. Heterologous co-expression of a monooxygenase and an epoxide hydrolase in *E. coli* allows whole cell conversion of C2-C4 alkenes for the formation of ethylene glycol, 1,2-propanediol, 1,2-butanediol, and 2,3-butanediol at ambient temperature and pressure in one pot. Increasing intracellular NADH supply via addition of formate and a formate dehydrogenase increases ethylene glycol production titers, resulting in an improved productivity of 9 mg/L/h and a final titer of 250 mg/L. This represents a novel biological method for GTL conversion of alkenes to industrially valuable diols.

1. Introduction

Gas-to-liquid (GTL) technologies convert gaseous substrates, typically methane, into liquid chemicals. Current industrial GTL processes employ catalysts with high heat and pressure (Haynes and Gonzalez, 2014). Heavy capital investment into large, high volume production facilities are required for economic viability, with the corollary requirement that large volumes of gas are constantly available (Haynes and Gonzalez, 2014). Such facilities are full of technical and economic challenges to build and maintain (Fei et al., 2014; Haynes and Gonzalez, 2014). A simpler and potentially cost effective alternative is to use engineered microbes as catalysts for GTL processes (Haynes and Gonzalez, 2014; Hu et al., 2016). A biological GTL system can operate at a smaller scale, at ambient temperatures, and with lower capital costs than conventional GTL systems. This would enable deployment of this technology to remote regions and allow capture of “stranded” gasses, which are an underutilized resource (Fei et al., 2014; Haynes and Gonzalez, 2014). Microorganisms can, in principle, achieve higher conversion efficiency (and substrate selectivity) than conventional GTL because of the enzymatic nature of the conversions (Fei et al., 2014). These factors make a biological GTL system potentially more economically and environmentally attractive than a conventional chemical GTL system (Fei et al., 2014; Haynes and Gonzalez, 2014).

Vicinal diols have broad applications as chemical feedstocks and fuels. They are produced industrially, largely from petroleum through a series of steps including cracking to alkenes, alkene epoxidation, and hydrolysis (Sabra et al., 2016). Ethylene glycol is of particular interest due to its many uses such as polyester fibers, PET (polyethylene terephthalate) plastics, and antifreeze. In 2010, 20 million metric tons of ethylene glycol was produced globally, with an estimated 5-10% increase in annual consumption (Yue et al., 2012). Industrially, ethylene glycol

production suffers from low specificity (~80%) for epoxidation by O₂, relatively poor conversion to monoethylene glycol (~90%), and high water and energy requirements (Rebsdatt and Mayer, 2000). This process presents an opportunity for improvement by biological catalysis, since it can potentially overcome all of these shortcomings. Furthermore, biological production can occur at ambient pressure and temperature and without the requirement of harsh chemical catalysts.

Larger diols (C₃-C₄) are also valuable (Burgard et al., 2016; Nakamura and Whited, 2003). For example, 1,2-propanediol is used in the food, pharmaceutical, cosmetic, and unsaturated polyester industries (Sullivan, 2000). It is also produced via alkene epoxidation and hydrolysis. Butanediols such as 2,3-butanediol serve as important feedstocks for rubbers, plastics, polymers, pharmaceuticals and insecticides (Gräfe et al., 2000). Ethylene glycol, 1,2-propanediol, 1,3-propanediol, 1,4-butanediol and 2,3-butanediol have been biologically produced from sugars (Burgard et al., 2016; Cam et al., 2015; Chen et al., 2016; Nakamura and Whited, 2003; Pereira et al., 2016; Pereira et al., 2015; Sabra et al., 2016).

Some microorganisms naturally metabolize gasses. For example, methanogens generate and methanotrophs catabolize methane, diazotrophs fix nitrogen gas, and photosynthetic organisms fix carbon dioxide (Desai and Atsumi, 2013; Kalyuzhnaya et al., 2015). This study demonstrates that *E. coli* can be engineered to directly metabolize gaseous C₂-C₄ alkenes to the corresponding diols, which are stable liquids under ambient conditions.

E. coli was chosen as a biological host because it is genetically tractable with many engineering tools readily available. The alkene metabolizing pathway was constructed with two heterologous enzymes. The first enzyme is a monooxygenase (MO) that converts the alkene to an epoxide, which is subsequently converted to a diol by an epoxide hydrolase (EH) from *Agrobacterium radiobacter* AD1 (Rink et al., 1997) (**Fig. 1A**). This combination of enzymes

converts gaseous C2-C4 alkenes (ethylene, propylene, 1-butene, cis-2-butene, and trans-2-butene) into the corresponding diols (ethylene glycol, 1, 2-propanediol, 1, 2-butanediol, *R,R*-/ *S,S*-2,3-butanediol, and meso-2, 3-butanediol).

2. Materials and Methods

2.1. Reagents

All enzymes were purchased from New England Biolabs. All synthetic oligonucleotides and DNA sequencing services were provided by Euforins. Chemicals for gas chromatography (GC) and High Performance Liquid Chromatography (HPLC) standards were purchased from Sigma Aldrich. Ethylene gas was purchased from Air Gas. Propene, 1-butene, cis and trans 2-butene, ethylene oxide and propylene oxide were purchased from Sigma Aldrich. 1,2-Epoxybutane was purchased from TCI America. cis and trans 2,3-Epoxybutane were purchased from Acros Organics.

2.2. Plasmid construction

All plasmids and primers are listed in **Table S1** and **Table S2**, respectively. The pAL1219 plasmid was constructed using a 300 bp fragment from pAL1354 and a 1.8 kbp fragment from pSA69 (Atsumi et al., 2008) cut with AatII and AvrII. All other plasmids were constructed using sequence and ligation-independent cloning (SLIC) (Li and Elledge, 2007). The pAL1220 plasmid was constructed using two fragments; the backbone was amplified from pZE12-luc (Lutz and Bujard, 1997) with primers SD11/YT18 and the gene encoding TOM V106A (Canada et al., 2002) was amplified with primers SD177/YT872. The pAL1293 plasmid was constructed using three fragments; the backbone was amplified from pZE12-luc with primers YT40/YT18 and the gene encoding toluene-4-monooxygenase (T4MO) (Tao et al., 2004) from *Pseudomonas*

mendocina KR1 was amplified with primers SD189/SD191 and primers SD187/SD190. The pAL1307 plasmid was constructed similarly to pAL1293 except that the gene encoding T4MO G103S/A107T (Tao et al., 2004) was used. The pAL1354 plasmid was constructed using two fragments amplified from pZE12-luc with primers SD138/SD194 and primers SD12/SD139. The pAL1439 plasmid was constructed using two fragments; the backbone was amplified from pSA69 (Atsumi et al., 2008) with primers YT5/YT6 and the epoxide hydrolase (*echA*) (Rink et al., 1997) gene, which was synthesized with an optimized codon usage for *E. coli* by Life Technologies, was amplified with primers SD178/SD179. The pAL1434 plasmids were constructed using two fragments; the backbone amplified from pZE12-luc (Lutz and Bujard, 1997) with primers YT40/YT18 and the gene encoding P450 BM3 910A (Meinhold et al., 2005) was amplified with primers SD210/SD211. pAL1456 was constructed using two fragments; backbone amplified from pAL1439 with primers SD199/SD200 and *fdh* from *Candida boidinii* (Shen et al., 2011) amplified with primers SD201/SD202. All plasmids are verified by restriction enzyme digest and sequencing.

2.3. NAD⁺/NADH assay

The NAD⁺/NADH assay was performed by the BioAssay Systems EnzyChrom NAD⁺/NADH assay kit (E2ND-100) kit using the manufacturer's protocol.

2.4. FDH Assay

Cells (6.4 mL) were harvested at 4°C, 2,000 g for 10 min. They were resuspended in 200 µL of 10 mM sodium phosphate buffer containing 100 mM beta-mercaptoethanol (BME). Cells were centrifuged once more, resuspended to a final volume of 200 µL in the same buffer as above and lysed using a mini bead beater 8 (BioSpec Productis, Inc.) by 4 rounds shaking, 45 seconds each. The crude lysate was centrifuged at 16,000 g for 30 min at 4°C. The cell lysate was mixed with

10 mM sodium phosphate buffer (pH 7.5), 1.67 mM NAD⁺, 167 mM formate, and 100 mM BME and absorbance was immediately read at 340 nm to detect NADH formation. NADH was calculated using an extinction coefficient of 6220 M⁻¹ cm⁻¹ (Schute et al., 1976).

2.5. Strain preparation

Strains were grown in LB overnight at 37°C with the appropriate antibiotics: ampicillin (200 µg/mL), kanamycin (50 µg/mL). Production experiments were carried out using a Modified Hutner's Mineral Base (MSB) Medium (Stanier et al., 1966), which consists of the following: 40 mM phosphate buffer, 7.57 mM (NH₄)₂SO₄, 0.52 M N(CH₂COOH)₃, 1.25 mM KOH, 1.16 mM MgSO₄, 0.3 mM CaCl₂•2H₂O, 0.72 µM (NH₄)₆Mo₇O₂₄•4H₂O, 4 µM EDTA, 23 µM FeSO₄•7H₂O, 51 µM MnSO₄•H₂O, 1.25 µM CuSO₄•5H₂O, 0.68 µM Co(NO₃)₂•6H₂O, 0.23 µM Na₂B₄O₇•10H₂O. Overnight cultures were inoculated 1% in MSB medium containing 5 g/L yeast extract and 50 g/L glucose. Cells were grown at 37°C, with shaking at 250 RPM. Once OD₆₀₀ reached ~0.4, gene expression was induced with 1 mM isopropyl-β-D-thio-galactoside (IPTG). At this point, cells were shifted to 30°C at 250 RPM for overnight growth. Subsequently, the cells were harvested at 1,500 g for 10 min and resuspended in MSB medium without glucose or yeast extract. The samples are centrifuged once more as above and then concentrated 3-fold to OD₆₀₀ 6 ± 1 in MSB medium.

2.6. Ethylene oxide and diol production

A 750 µL sample was placed into a 3 mL rubber capped tube (BD vacutainer). Samples were bubbled with ethylene for 5 s with 1.5% ethylene (1 psi). For 100% ethylene, propylene, 1-butene, cis-2-butene, and trans-2-butene, samples were bubbled for 1 s. Subsequently, samples were incubated at 30°C for 24 h while lying flat on a shaker (350 RPM). Ethylene oxide samples

were spun down at 16,000 g and the cell supernatant was used for GC analysis. Diol samples were analyzed via HPLC.

Epoxide hydrolase assay

A 750 μ L sample was introduced into 3 mL rubber capped tubes. Epoxide was added to a final concentration of 5mM and the sample was incubated at 30°C for 24 h in a shaker (350 RPM). After incubation, samples were centrifuged at 16,000 g and the supernatant was used for GC analysis.

2.7. Ethylene glycol production in the presence of formate

Strains were condensed to 1.5 mL (starting OD₆₀₀ ~7) from 6.4 mL of induced cultures and put in 10 mL rubber capped tubes. Initial formate concentration was 5 mM, and 1.5% ethylene was bubbled in at 1psi for 20 s. The sample was put at 30°C in a microplate shaker (350 RPM). After 24 h, 5 mM formate was added to the samples and ethylene was introduced as described above. Samples were taken at 24 and 48 h, spun down at 16,000 g and cell supernatant was used for HPLC analysis.

2.8. Ethylene glycol production at high cell density

Strains were condensed to 1 mL (starting OD₆₀₀ 57 for **Strain TOF**) from 120 mL of induced cultures and put in a 10 mL rubber capped tube. Initial formate concentration was 8.3 mM, and 1.5% ethylene bubbled in at 1psi for 20 seconds. The tube was subsequently put at 30°C in a microplate shaker (350 RPM). After 6, 12, and 24 h, 5 mM formate was added to the samples and ethylene was introduced as described above. Samples (200 μ L) spun down at 16,000 g and cell supernatant was used for HPLC analysis at 6 and 48 h.

2.9. GC analysis

GC-FID was used to quantify oxides. The GC system is a GC-2010 with an AOC-20 S auto sampler and AOC-20i Auto Injector (Shimadzu). The column used was a DB-Wax capillary column (30 m length, 0.32-mm diameter, 0.50- μ m film thickness) (Agilent Technologies). The GC oven temperature was begun at 40°C, increased temperature at 45°C per minute to 225°C and held for 3 minutes, and the FID detector was held at 330°C. The injection volume was 0.5 μ L, injected at a 15:1 split ratio. Helium was used as the carrier gas. 1-Pentanol (100 mg/L) was used as an internal standard.

2,3-Butanediol from epoxide feeding was detected using the HP-5 column (30 m length, 0.25 mm diameter, 0.25 μ m film thickness). The GC oven temperature began at 70°C and increased to 150°C at 40°C per minute, and the FID detector was held at 330°C. Injection volume was 0.5 μ L, injected at a 50:1 split ratio. Helium was used as the carrier gas. 1-Pentanol (100 mg/L) was used as an internal standard.

2.10. HPLC Analysis

To measure formate and diol formation, cell culture supernatant was measured using a 20A high-performance liquid chromatography (HPLC from Shimadzu) equipped with a differential refractive index detector (RID) 10A and an Aminex fast acid analysis column (Biorad). The mobile phase contained 5 mM H₂SO₄, at a flow rate of 0.6 mL/min at 60°C.

3. Results

3.1. Conversion of alkenes to diols

E. coli does not naturally assimilate gaseous alkenes, therefore heterologous enzymes that convert C₂-C₄ alkenes to the corresponding oxides were identified. Epoxides are toxic to living organisms and relatively unstable (Ehrenberg and Hussain, 1981). It was therefore expected that

efficient microbial production of the diols would require rapid *in vivo* conversion of the nascent epoxide to the diol so that epoxide buildup, and thereby toxicity, does not occur. This can be achieved by coexpression of an EH that rapidly converts epoxides to diols (**Fig. 1A**).

An EH encoded by *echA* from *A. radiobacter* AD1 was identified (Rink et al., 1997). It has been shown to be active *in vitro* toward a variety of epoxides including ethylene oxide, propylene oxide and 1,2-epoxybutane (Rink et al., 1997). The EH was expressed well in *E. coli* harboring a synthetic *echA* gene, which was codon optimized for *E. coli*. The *in vivo* activity was tested by feeding 5 mM of each epoxide to *E. coli* expressing *echA*. The corresponding diols were observed, confirming that EchA is active inside *E. coli* (**Fig. 1B**). From 5 mM ethylene oxide (220 mg/L), ~4 mM (184 mg/L) ethylene glycol was produced, while from 5 mM propylene oxide (290 mg/L), 1.3 mM (76 mg/L) of 1,2-propanediol was formed (**Fig. 1B**). These results are consistent with previous results showing that EchA has better activity on ethylene oxide than propylene oxide *in vitro* (Rink et al., 1997). The *in vitro* activity of EchA was reported to be better with 1,2-epoxybutane than ethylene oxide (Rink et al., 1997), but only 0.8 mM 1,2-butanediol (56 mg/L) was formed from 5 mM 1,2-epoxybutane (361 mg/L) (**Fig. 1B**). Additionally, 0.2 mM (17 mg/L) and 0.06 mM (6 mg/L) 2,3-butanediol were produced from 5 mM cis-2,3-epoxybutane and trans-2,3-epoxybutane (361 mg/L), respectively (**Fig. 1B**). Decreasing diol conversion with increasing epoxide size may indicate a diffusional barrier to catalysis since *in vitro* data (Rink et al., 1997) do not show the same trend (**Fig. 1B**). Regardless, EchA accepts a variety of epoxide substrates and was chosen as the EH for this pathway.

Next, enzymes that can epoxidize alkenes were identified (**Fig. 2**). Some MOs have been reported to perform this reaction, including P450 BM3 mutants (Alcalde et al., 2004; Fasan et al., 2007; Meinhold et al., 2005), toluene-4-monooxygenase (T4MO) mutants (Tao et al., 2004), and

toluene *ortho*-monooxygenase (TOM) mutants (Canada et al., 2002; Carlin et al., 2015). Four candidates TOM (V106A) (Canada et al., 2002), T4MO wild type, T4MO (G103S/A107T) (Tao et al., 2004) and P450 BM3 9-10A (Meinhold et al., 2005) were chosen for further characterization based on reported activities and preliminary screening.

During the initial screening in rich media (Luria-Bertani Broth and Terrific Broth), inconsistent titers and culture pigmentation were observed within identical replicates. MOs can convert tryptophan in the rich media into indole, which spontaneously generates colored compounds (Canada et al., 2002). Competition between aromatic amino acids and ethylene may cause the inconsistent results. However, strains harboring the MOs were unable to grow in M9 minimal medium (Desai et al., 2015). Another minimal medium, modified Hutner's mineral base (MSB) (Stanier et al., 1966), was identified which has a higher iron content than M9 based medium. It was speculated that this medium would be beneficial for MO activity since these enzymes require iron for catalysis. MSB medium enabled strains containing MOs to grow and no culture pigmentation was observed. Thus, MSB was used for further characterization.

The promiscuity of the MOs was explored since EH can accept a variety of epoxides in *E. coli* (**Fig. 1B**). The epoxide products of the MO reactions with alkenes are unstable, toxic, and volatile which makes their yields difficult to directly quantify. Therefore, the *in vivo* MO reactions were coupled to EH, yielding the diol, which is stable and easily quantified.

Diol formation from C2-C4 alkenes by strains containing both MO and EH is presented in **Fig. 2**. Ethylene, propene, 1-butene, cis-2-butene, and trans-2-butene were tested for diol formation by introducing 100% of each gas for 1 s into the headspace of a 3 mL vacuum tube. These alkenes were chosen because the corresponding diols are important industrial chemicals used in a variety of applications (Sabra et al., 2016).

When cells were introduced to 100% ethylene, ethylene glycol was produced by three of the four strains harboring MOs and EH (**Fig. 2B**). The strain harboring T4MO (**Strain T4**) produced 30 mg/L ethylene glycol, while the strains harboring TOM (V106A) (**Strain TO**) and T4MO (G103S/A107A) (**Strain T4m**) produced slightly less ethylene glycol (19 and 24 mg/L). Different ethylene gas percentages were screened for their effect on ethylene glycol formation. When 1.5% ethylene was used, ethylene glycol was produced up to 60 mg/L (**Fig. S1**). With propene as a substrate, **Strains TO** and **T4** produced 10 and 11 mg/L of 1,2-propanediol, respectively. Slightly less 1,2-propanediol (6 mg/L) was observed with **Strain T4m** (**Fig. 2C**). 1-Butene was converted to 1,2-butanediol with yields of 3 mg/L, 4 mg/L, and 5 mg/L by **Strains TO**, **T4**, and **T4m**, respectively (**Fig. 2D**). *cis*-2-Butene was converted to 11 mg/L *R,R*- and/or *S,S*-2,3-butanediol by **Strains T4** and **T4m**. Quantification methods used in this study cannot discriminate *R,R*- and *S,S*-2,3-butanediol due to the low titer. Very little conversion of *cis*-2-butene was observed for **Strain TO** (**Fig. 2E**). *trans*-2-Butene was converted to meso-2,3-butanediol (**Fig. 2F**). **Strains TO** and **T4m** produced 16 and 13 mg/L meso-2,3-butanediol, respectively, while **Strain T4** produced more (29 mg/L) (**Fig. 2F**).

The strain harboring P450 BM3 9-10A did not show conversion to any of the diols in this condition. The P450 BM3 9-10A may require more oxygen than other MOs (Meinhold et al., 2005). Additionally, P450s generally require the heme precursor δ -aminolevulinic acid (Meinhold et al., 2005) be added to the cell culture for sufficient heme formation and proper enzyme folding. This extra step is not required for the other MOs tested in this study, and may also contribute to the lack of observed activity.

Strains harboring the MO genes but not the EH gene were tested for ethylene oxide production from ethylene (**Fig. 2G**). Strains harboring TOM (V106A), T4MO, and T4MO

(G103S/A107A) produced ethylene oxide (**Fig. 2G**), confirming ethylene glycol production occurs via ethylene oxide.

3.2. Effect of NADH Supply on Ethylene Glycol Production

The production of diols from gaseous alkene in *E. coli* demonstrated above is promising but the titers are low. From **Fig. 1B**, it was concluded that EH is unlikely to be the bottleneck since it converts ethylene oxide to ethylene glycol rapidly. Therefore, it was hypothesized that the intracellular NADH supply limits MO turnover and thereby epoxide formation from ethylene. NADH can be regenerated from NAD⁺ if cells are continuously fed rich medium or sugars such as glucose. However, doing so is not beneficial overall since it increases production costs. Instead, an abundant and inexpensive substrate, formate, was chosen as a source of reducing equivalents. Formate can be formed from CO₂ via electrolysis (Li et al., 2012), and is converted to NADH and CO₂ by formate dehydrogenase (FDH) (Schute et al., 1976). The released CO₂ can be recycled to formate via electrolysis (Li et al., 2012). Adding an *fdh* gene to the present strains and feeding formate should increase intracellular NADH and thereby ethylene glycol titers. To test this hypothesis, the *fdh* gene from *Candida boidinii* (Shen et al., 2011) was introduced into **Strains TO** and **T4** (**Fig. 2**) to give **Strains TOF** and **T4F**, respectively. FDH activity in **Strain TO** was confirmed (**Fig. 3A**).

Strains TOF and **T4F** were used in a 48 h experiment where ethylene and 5 mM formate were fed at 0 and 24 h to determine if formate and *fdh* were beneficial to ethylene glycol production (**Fig. 3**). In the presence of both *fdh* and formate, 140 mg/L ethylene glycol was observed after 48 h for **Strain TOF**, while only 40 mg/L ethylene glycol was produced in **Strain TO** without formate and *fdh*, corresponding to a 3.5-fold improvement (**Fig. 3**). For **Strain T4**

(without *fdh*), the ethylene glycol titer with formate was 53 mg/L after 48 h, while **Strain T4F** in the presence of formate gave 105 mg/L, corresponding to a 2-fold improvement (**Fig. 3B**). However, the ethylene glycol titer of **Strain T4F** did not change significantly with formate supplementation. The intracellular NAD⁺/NADH ratio was measured to clarify the relationship between intracellular NADH and ethylene glycol productions (**Fig. 3C**), but no significant trends were observed.

In separate experiments, ethylene and formate were supplied more frequently to the cells. In one experiment, in 70 mL test tubes, 1.5 % ethylene was bubbled for one hour every 12 h with 5 mM formate supplementation, but this did not improve ethylene glycol titers (data not shown). To improve gas exchange, the cells were placed in a petri dish (to increase the gas-liquid interface) inside of a box pressurized with 1.5 % ethylene, but no improvement in ethylene glycol titers was observed (data not shown). Finally, ethylene and 5 mM formate were fed at 6, 12 and 24 h during a 48 h production period using a 10 mL glass blood serum vial. After 6 h, **Strains TOF** produced 80 mg/L (**Fig. 4**). Utilizing this method, 250 mg/L ethylene glycol was achieved after 48 h (**Fig. 4**), doubling the highest titer obtained from **Fig. 3**.

4. Discussion

This study demonstrates a novel, sustainable route for production of industrially important diols. These compounds are routinely utilized as antifreeze agents, solvents, and polymer precursors (Sabra et al., 2016). The active development of microbial diol production (Burgard et al., 2016; Nakamura and Whited, 2003) prognosticates a future with biologically based industrial production of diols. Previously developed biological routes to diols use glucose or glycerol as the feedstock (Burgard et al., 2016; Nakamura and Whited, 2003), while diol production from gaseous alkenes demonstrated herein is an alternative to sugar feedstocks. It is

also a first step toward biological GTL conversion of underutilized feedstocks such as stranded natural gas which can be converted into ethylene.

The two enzymes chosen here to construct the novel metabolic route proved broadly useful. The EH from *A. radiobacter* AD1 encoded by *echA* has activity on a wide range of substrates. It can hydrolyse ethylene oxide, propylene oxide, and 1,2-epoxybutane in *E. coli* (**Fig. 1B**). Previously no conversion of cis-2,3-epoxybutane to the diol was observed *in vitro* (Rink et al., 1997). However, both cis-2,3-epoxybutane and trans-2,3-epoxybutane were successfully converted to 2,3-butanediol *in vivo* by *E. coli* expressing *echA* (**Fig. 1B**). **Strains TO, T4, and T4m**, which contain genes for different MOs, showed activity on several alkenes (ethylene, propylene, 1-butene, cis-2-butene, trans-2-butene) as evidenced by the formation of the corresponding diols (**Fig. 2**). These unprecedented results demonstrate the promiscuity both for the MOs to form epoxides and the EH to form the respective diols.

Titers for 2,3-butanediol were greater from trans-2-butene than cis-2-butene, even though EH has less activity on the trans than the cis isomer (**Fig. 1B and 2F**). It was hypothesized that the MOs have greater activity with trans-2-butene than with the cis isomer. This would result in higher epoxybutane production in the suspected rate-limiting conversion of alkene to epoxide with the trans compared to the cis isomer, and, therefore, higher overall meso-2,3-butanediol titers.

Several attempts were made to improve the titer for ethylene glycol. A limiting factor was speculated to be low intracellular NADH concentrations with concomitant low MO activity, since the EH efficiently converts ethylene oxide to ethylene glycol (**Fig. 1**). To increase NADH supply, FDH was introduced into ethylene glycol forming strains and formate was included in the production medium. **Strain TOF** in the presence of 5 mM formate showed >50% improved

ethylene glycol titer compared to **Strain TO** (no *fdh*) (**Fig. 3B**). Without formate addition to **Strain TOF**, ethylene glycol titer does not increase after 24 h, suggesting that a supply of reducing equivalents is essential for continuous ethylene glycol production (**Fig. 3B**). With formate addition to **Strain TOF**, the ethylene glycol titer increases ~30% from 24 h to 48 h. A number of different culture conditions were tested to explore the interaction between substrate and NADH supply. Supplementation with ethylene and formate at 6, 12, 24, and 48 h doubled the ethylene glycol titer (**Fig. 4**). After 6 h and 72 h, **Strain TOF** produced 52 mg/L (productivity of 9 mg/L/h) and 250 mg/L ethylene glycol, respectively (**Fig. 4**). Thus, a source of reducing equivalents for regenerating NADH is crucial for continuous ethylene glycol production. This follows from the fact that one equivalent of NADH is consumed in the MO reaction for every equivalent of diol formed.

The diol titers and productivities reported here are low compared to titers achieved with sugars as a substrate (Pereira et al., 2016; Pereira et al., 2015). Nevertheless, the results presented in this study are encouraging when compared to other GTL systems. Methanotrophs, a natural GTL platform, can be engineered to convert methane into liquid chemicals (Haynes and Gonzalez, 2014), yet working with them is challenging because they are difficult to manipulate genetically, grow slowly, and have low yields (Henard et al., 2016). Despite these challenges, one group demonstrated lactate production (8 mg/L/h) from a methanotrophic bacterium (*Methylobaculum buryatense*) using a bioreactor (Henard et al., 2016). Another group engineered an archaeal methanogen to produce acetate (590 mg/L at 5 mg/L/h) with methane as a sole carbon source (Soo et al., 2016). Photosynthesis is nature's most prevalent gas capturing system, harnessing CO₂ to produce biomass. Several groups have metabolically engineered cyanobacteria for chemical production from CO₂ (Desai and Atsumi, 2013). For example, with

Synechococcus elongatus PCC 7942, a productivity of 36 mg sucrose/L/h from CO₂ was achieved (Ducat et al., 2012). Additionally, *S. elongatus* has been engineered to produce 2,3-butanediol with a productivity of 5 mg/L/h (Oliver et al., 2013).

Converting methane or CO₂ into complex chemicals requires carbon-carbon bond formation which is an energy intensive process. By providing *E. coli* with gaseous alkenes, this difficult and energy intensive step is by-passed, allowing facile production of valuable diols (Sabra et al., 2016). The best ethylene glycol productivity reported here is 9 mg/L/h (**Fig. 4**), which surpasses the biological production of diols from cyanobacteria and acetate from methanotrophs.

The pathway presented here is industrially relevant and contributes to the development of industrially viable metabolic routes for conversion of gaseous substrates into valuable liquid chemicals. Since Fisher-Tropsch technologies to convert methane to liquid fuels require complex facilities, with associated high costs, many companies (*e.g.*, LanzaTech, Intrexon, Calysta, etc) are acknowledging the benefits of biological production and investing in its development. Further optimization, particularly of the MO activity toward alkene epoxidation, may develop this new pathway into a viable industrial process.

Acknowledgments

This work was supported by the REMOTE program of the ARPA-E (Award: DE-AR0000429). We would like to thank Thomas K. Wood (Pennsylvania State University) for providing TOM and T4MO mutants and Frances H. Arnold (California Institute of Technology) for providing P450 BM3 mutants. We are grateful to Rebecca E. Paraless, D. Alex Carlin and Justin B. Siegel for insightful discussions.

References

- Alcalde, M., et al., 2004. Colorimetric high-throughput assay for alkene epoxidation catalyzed by cytochrome P450 BM-3 variant 139-3. *J Biomol Screen.* 9, 141-146.
- Atsumi, S., et al., 2008. Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature.* 451, 86-89.
- Burgard, A., et al., 2016. Development of a commercial scale process for production of 1,4-butanediol from sugar. *Curr Opin Biotechnol.* 42, 118-125.
- Cam, Y., et al., 2015. Engineering of a Synthetic Metabolic Pathway for the Assimilation of (d)-Xylose into Value-Added Chemicals. *ACS Synth Biol.* doi:10.1021/acssynbio.5b00103.
- Canada, K. A., et al., 2002. Directed evolution of toluene ortho-monooxygenase for enhanced 1-naphthol synthesis and chlorinated ethene degradation. *J Bacteriol.* 184, 344-349.
- Carlin, D. A., et al., 2015. Biocatalytic conversion of ethylene to ethylene oxide using an engineered toluene monooxygenase. *Chem Commun (Camb).* 51, 2283-2285.
- Chen, Z., et al., 2016. Metabolic engineering of *Corynebacterium glutamicum* for the *de novo* production of ethylene glycol from glucose. *Metab Eng.* 33, 12-18.
- Desai, S. H., Atsumi, S., 2013. Photosynthetic approaches to chemical biotechnology. *Curr Opin Biotechnol.* 24, 1031-1036.
- Desai, S. H., et al., 2015. Isobutanol production from cellobionic acid in *Escherichia coli*. *Microb Cell Fact.* 14, 52.
- Ducat, D. C., et al., 2012. Rerouting carbon flux to enhance photosynthetic productivity. *Appl Environ Microbiol.* 78, 2660-2668.
- Ehrenberg, L., Hussain, S., 1981. Genetic toxicity of some important epoxides. *Mut Res.* 86, 1-113.

396 Fasan, R., et al., 2007. Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting
 397 nativelylike catalytic properties. *Angew Chem Int Ed Engl.* 46, 8414-8418.
 398 Fei, Q., et al., 2014. Bioconversion of natural gas to liquid fuel: opportunities and challenges.
 399 *Biotechnol Adv.* 32, 596-614.
 400 Gräfe, H., et al., 2000. Butanediols, Butenediol, and Butynediol. *Ullmann's Encyclopedia of*
 401 *Industrial Chemistry.* 6, 407-414.
 402 Haynes, C. A., Gonzalez, R., 2014. Rethinking biological activation of methane and conversion
 403 to liquid fuels. *Nat Chem Biol.* 10, 331-339.
 404 Henard, C. A., et al., 2016. Bioconversion of methane to lactate by an obligate methanotrophic
 405 bacterium. *Sci Rep.* 6, 21585.
 406 Hu, P., et al., 2016. Integrated bioprocess for conversion of gaseous substrates to liquids. *Proc*
 407 *Natl Acad Sci U S A.* 113, 3773-3778.
 408 Kalyuzhnaya, M. G., et al., 2015. Metabolic engineering in methanotrophic bacteria. *Metab Eng.*
 409 29, 142-152.
 410 Li, H., et al., 2012. Integrated electromicrobial conversion of CO₂ to higher alcohols. *Science.*
 411 335, 1596.
 412 Li, M. Z., Elledge, S. J., 2007. Harnessing homologous recombination *in vitro* to generate
 413 recombinant DNA via SLIC. *Nat Methods.* 4, 251-256.
 414 Lutz, R., Bujard, H., 1997. Independent and tight regulation of transcriptional units in
 415 *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements.
 416 *Nucleic Acids Res.* 25, 1203-10.
 417 Meinhold, P., et al., 2005. Direct conversion of ethane to ethanol by engineered cytochrome
 418 P450 BM3. *Chembiochem.* 6, 1765-8.

419 Nakamura, C. E., Whited, G. M., 2003. Metabolic engineering for the microbial production of
 420 1,3-propanediol. *Curr Opin Biotechnol.* 14, 454-459.
 421 Oliver, J. W., et al., 2013. Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. *Proc*
 422 *Natl Acad Sci U S A.* 110, 1249-1254.
 423 Pereira, B., et al., 2016. Efficient utilization of pentoses for bioproduction of the renewable two-
 424 carbon compounds ethylene glycol and glycolate. *Metab Eng.* 34, 80-87.
 425 Pereira, B., et al., 2015. Engineering a novel biosynthetic pathway in *Escherichia coli* for
 426 production of renewable ethylene glycol. *Biotechnol Bioeng.* 113, 376-383.
 427 Rebsdatt, S., Mayer, D., 2000. Ethylene Glycol. *Ullmann's Encyclopedia of Industrial Chemistry.*
 428 13, 531-544.
 429 Rink, R., et al., 1997. Primary structure and catalytic mechanism of the epoxide hydrolase from
 430 *Agrobacterium radiobacter* AD1. *J Biol Chem.* 272, 14650-14657.
 431 Sabra, W., et al., 2016. Microbial cell factories for diol production. *Adv Biochem Eng*
 432 *Biotechnol.* 155, 165-97.
 433 Schute, H., et al., 1976. Purification and properties of formaldehyde dehydrogenase and formate
 434 dehydrogenase from *Candida boidinii*. *Eur J Biochem.* 62, 151-160.
 435 Shen, C. R., et al., 2011. Driving forces enable high-titer anaerobic 1-butanol synthesis in
 436 *Escherichia coli*. *Appl Environ Microbiol.* 77, 2905-2915.
 437 Soo, V. W., et al., 2016. Reversing methanogenesis to capture methane for liquid biofuel
 438 precursors. *Microb Cell Fact.* 15, 11.
 439 Stanier, R. Y., et al., 1966. The aerobic pseudomonads: a taxonomic study. *J Gen Microbiol.* 43,
 440 159-271.

441 Sullivan, C. J., 2000. Propanediols. Ullmann's Encyclopedia of Industrial Chemistry. 30, 233-
442 240.

443 Tao, Y., et al., 2004. Altering toluene 4-monooxygenase by active-site engineering for the
444 synthesis of 3-methoxycatechol, methoxyhydroquinone, and methylhydroquinone. J
445 Bacteriol. 186, 4705-13.

446 Yue, H., et al., 2012. Ethylene glycol: properties, synthesis, and applications. Chem Soc Rev. 41,
447 4218-44.

448

450

451 **Figure 1. Characterizing enzymes for diol production.** A. Reaction scheme for alkene to diol
 452 production with ethylene glycol shown as an example B. *In vivo* conversion of individually fed
 453 epoxides to diols. Here, 5 mM of different epoxides were fed to strains expressing the epoxide
 454 hydrolase gene. Numbers below epoxides represent mass concentration (mg/L) for the initial 5
 455 mM solution, while numbers above the bars represent diol formation. In a negative control
 456 (empty vector), no detectable peaks were observed. N=3; error bars represent standard
 457 deviations.

458

Figure 2. Diol production from different gaseous alkenes. Strains expressing both the monooxygenase (MO) and epoxide hydrolase (EH) genes were bubbled with different gaseous alkenes, and diol production was determined after 24 h. Pure alkene gas was introduced into the headspace for 1 second. **A.** Monooxygenase numbering. **B.** Ethylene glycol (EG) production from ethylene **C.** 1,2-Propanediol production (PDO) from propene. **D.** 1,2-Butanediol production (12BDO) from 1-butene. **E.** *R,R*- and/or *S,S*-2,3-Butanediol (23BDO) from cis-2-butene. **F.** meso-2,3-Butanediol production (23BDO) from trans-2-butene. **G.** Ethylene oxide production from ethylene in strains without EH. N=3; error bars represent standard deviations.

Figure 3. Ethylene glycol production with formate and FDH. **A.** FDH enzyme activity at 24 h. **B.** Strains, with and without FDH, and with and without supplemented formate were monitored for ethylene glycol production over 48 h. **C.** Intracellular ratio of NAD⁺ to NADH concentrations after 48 h. EH, epoxide hydrolase; FDH, formate dehydrogenase; OD, optical density at time zero; MO, monooxygenase. N=3; error bars represent standard deviations.

Figure 4. Ethylene glycol production from high cell densities. Ethylene glycol production from Strain TOF by feeding 10mM formate and ethylene at 0, 6, 12, 24, 48, and 72 h. N=3; error bars represent standard deviations. Starting OD₆₀₀ was 57.