Metabolic Engineering Communications 3 (2016) 211–215

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

# Enhanced fatty acid production in engineered chemolithoautotrophic bacteria using reduced sulfur compounds as energy sources

Harry R. Beller [a,b,*], Peng Zhou [b,1], Talia N.M. Jewell [b], Ee-Been Goh [a,c], Jay D. Keasling [a,c,d,e]

[a] Joint BioEnergy Institute, 5885 Hollis Avenue, Emeryville, CA, USA
[b] Earth and Environmental Sciences, Lawrence Berkeley National Laboratory (LBNL), Berkeley, CA, USA
[c] Biological Systems and Engineering, LBNL, Berkeley, CA, USA
[d] Departments of Chemical & Biomolecular Engineering and of Bioengineering, University of California, Berkeley, CA, USA
[e] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kogle Allé, DK2970 Hørsholm, Denmark

ARTICLE INFO

*Article history:*
Received 10 April 2016
Received in revised form
19 June 2016
Accepted 4 July 2016
Available online 5 July 2016

*Keywords:*
Chemolithoautotrophic
Sulfide
Fatty acids
*tesA*
*Thiobacillus denitrificans*

ABSTRACT

Chemolithoautotrophic bacteria that oxidize reduced sulfur compounds, such as $H_2S$, while fixing $CO_2$ are an untapped source of renewable bioproducts from sulfide-laden waste, such as municipal wastewater. In this study, we report engineering of the chemolithoautotrophic bacterium *Thiobacillus denitrificans* to produce up to 52-fold more fatty acids than the wild-type strain when grown with thiosulfate and $CO_2$. A modified thioesterase gene from *E. coli* ('*tesA*) was integrated into the *T. denitrificans* chromosome under the control of $P_{kan}$ or one of two native *T. denitrificans* promoters. The relative strength of the two native promoters as assessed by fatty acid production in engineered strains was very similar to that assessed by expression of the cognate genes in the wild-type strain. This proof-of-principle study suggests that engineering sulfide-oxidizing chemolithoautotrophic bacteria to overproduce fatty acid-derived products merits consideration as a technology that could simultaneously produce renewable fuels/chemicals as well as cost-effectively remediate sulfide-contaminated wastewater.



## 1. Introduction

Hydrogen sulfide in wastewater, for example, municipal or petroleum refinery hydrodesulfurization wastewater, is an untapped energy source for renewable chemicals and biofuels. Biological oxidation of hydrogen sulfide to sulfate by chemolithoautotrophic bacteria (reviewed by Shao et al. (2010)) has been studied as an alternative to expensive chemical treatment methods for sulfide, but the concept of coupling chemolithoautotrophic sulfide oxidation to simultaneous production of renewable chemicals or biofuels from $CO_2$ has not been discussed in the scientific literature, to our knowledge. The obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans* has been studied with respect to treatment of sulfide-containing wastewaters since the 1980s. In these bench-scale studies, *T. denitrificans* catalyzed sulfide oxidation with nitrate as the electron acceptor (Cardoso et al., 2006; Garcia-de-Lomas et al., 2007; Kleerebezem and Mendez, 2002; Manconi et al., 2007; Ongcharit et al., 1991; Shao et al., 2010; Sublette and Sylvester, 1987). The process could also be conducted with oxygen as the electron acceptor, but use of nitrate would be advantageous for several reasons: (1) denitrification of nitrate to $N_2$ (as is catalyzed by *T. denitrificans*; Beller et al., 2006) would be a way of remediating nitrate occurring in municipal wastewater facilities using nitrification, (2) denitrification would eliminate the need for costly aeration, and (3) hydrogen sulfide oxidation with denitrification produces 5-fold fewer protons per mole of sulfide than aerobic respiration, so would require less buffering. Engineering of *T. denitrificans* for generation of renewable products is feasible, as methods for genetic manipulation of this organism, including chromosomal integration and deletion, have been developed in recent years (Beller et al., 2012, 2013; Letain et al., 2007).

In this proof-of-principle study, we investigated whether *T. denitrificans* could be engineered to overproduce fatty acids while oxidizing a reduced sulfur compound as a sole electron donor, reducing nitrate as a sole electron acceptor, and fixing $CO_2$ as a sole carbon source. Specifically, we investigated whether the chromosomal integration of '*tesA*, a cytoplasmically directed acyl-

*Abbreviations:* ACP, acyl carrier protein; PCR, polymerase chain reaction

* Corresponding author at: Joint BioEnergy Institute, 5885 Hollis Avenue, Emeryville, CA, USA.
*E-mail address:* HRBeller@lbl.gov (H.R. Beller).
[1] Current address: Second Institute of Oceanography, State Oceanic Administration, China.

http://dx.doi.org/10.1016/j.meteno.2016.07.001

ACP thioesterase from *E. coli*, under the control of the kanamycin promoter ($P_{kan}$) or native promoters, would substantially increase fatty acid titer. We chose '*tesA* because this thioesterase has been shown to effectively deregulate fatty acid biosynthesis and consequently enhance fatty acid production in various bacteria, particularly *E. coli*, by hydrolyzing fatty acyl-ACPs that normally stringently regulate acetyl-CoA carboxylase (ACC) and, to a lesser extent, β-ketoacyl-ACP synthase III (FabH) and enoyl-ACP reductase (FabI) (Beller et al., 2015; Cho and Cronan, 1995; Müller et al., 2013; Steen et al., 2010).

## 2. Materials and methods

### 2.1. Bacterial strains, oligonucleotides, and reagents

Wild-type and mutant strains of *Thiobacillus denitrificans* and *E. coli* that were used in this study are listed in Table 1 and primers used for strain construction are listed in Table 2. The chemicals used in this study were of the highest purity available and were used as received. Highly purified water (18 MΩ resistance) obtained from a Milli-Q Biocel system (Millipore, Bedford, MA) was used to prepare all aqueous solutions described in this article.

### 2.2. Growth medium and cultivation conditions

All experiments described in this article were performed at 30 °C under strictly anaerobic conditions in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, Mich.) with a nominal gas composition of 85% $N_2$ – 10% $CO_2$ – 5% $H_2$. The glass and plastic materials used to contain or manipulate the cultures were allowed to degas in the glove box for at least one day before use. For liquid cultures, *T. denitrificans* strains were cultivated in anaerobic growth medium (pH ~7) described previously (Beller et al., 2012) that included the following compounds added at the concentrations specified in parentheses: $Na_2S_2O_3 \cdot 5H_2O$ (20 mM), $KNO_3$ (20 mM), $NH_4Cl$ (18.7 mM), $KH_2PO_4$ (14.7 mM), $NaHCO_3$ (30 mM), $MgSO_4 \cdot 7H_2O$ (3.25 mM), $CaCl_2 \cdot 2H_2O$ (0.05 mM), and anaerobic and sterile solutions of vitamins, trace elements, and selenite-tungstate prepared as described by Widdel and Bak (1992) (stock solutions 1, 4, 6, 7, and 8). Kanamycin (50 μg/mL) was added, as appropriate. Anaerobic techniques used in the preparation of growth medium and stock solutions are described elsewhere (Beller et al., 2012). Growth on solid medium was conducted under denitrifying conditions at 30 °C in an anaerobic glove box, as described in detail elsewhere (Beller et al., 2012). For wild-type *T. denitrificans* (ATCC 25259), a period of 5–10 days was required for isolated colonies to be of appropriate size for transfer to liquid medium.

For tests of fatty acid production, *T. denitrificans* strains were grown anaerobically in 200 mL of medium in 250-mL amber glass bottles sealed with polytetrafluoroethylene (PTFE) Mininert screw-cap valves (Sigma-Aldrich, St. Louis, MO) under static conditions in an anaerobic glove box.

**Table 2**
PCR primers used in this study.

| Primer | Sequence[a] (5′ – 3′) |
|---|---|
| ltesA-f | ATGGCGGACACGTTATTG |
| ltesA-r-*Eco*RI | GGGGAATTCTTATGAGTCATGATTTAC |
| Ptbd_2545f-*Hind*III | GGGAAGCTTTTTCCGTCGGCGTACCGATC |
| Ptbd_2545r-tesA | ATCAATAACGTGTCCGCCATGATCTTCCCATCCATCCGAT |
| Ptbd_2726f-*Hind*III | GGGAAGCTTTGAGCGCCGGGATCGAGAAGG |
| Ptbd_2726r-tesA | ATCAATAACGTGTCCGCCATTACAGTCTCCGTAGAGGTCG |
| kanP-f-*Hind*III | GGGAAGCTTAAAGCCACGTTGTGTCTC |
| tesA-f-kanP-r | CAGTAATACAAGGGGTGTTATGGCGGACACGTTATTG |
| Tbd_2545_KO1–1[b] | TACTCCTTGTCCAGCAGGTG |
| 2545UP-2-*Hind*III | GGGAAGCTTATCGCGGAACCTCTTTTCAT |
| KO3-*Eco*RI[b] | GCAGAATTCCAACCATCATCGATGAATTG |
| Tbd_2545_KO6–1[b] | TCGCGATCGCCTACATCGAC |

[a] Relevant restriction sites are underlined.
[b] Primer was previously used by Beller et al. (2013).

### 2.3. Engineering insertions into the T. denitrificans chromosome

A rapid, single-step gene replacement approach described previously (Beller et al., 2013) was used to make chromosomal insertions in *T. denitrificans*. Insertions were made in the Tbd_2545 gene (encoding a diheme *c*-type cytochrome of unknown function; Beller et al., 2006), which was replaced with the following: (a) a regulatory region including either $P_{kan}$ or the upstream region for Tbd_2545 (203 bp) or Tbd_2726 (218 bp) that includes the native promoters for these genes, (b) the *'tesA* gene, and (c) the kanamycin resistance (*kan*) gene together with its upstream region (119 bp). For the three engineered strains with *'tesA* insertions (strains Pkan, P2545, and P2726, with the $P_{kan}$, $P_{2545}$, or $P_{2726}$ promoters upstream of *'tesA*, respectively; Table 1), *'tesA* was placed at the ATG site of the gene replaced. To illustrate the approach, we describe below how this technique was used to replace the gene by homologous recombination with the promoter region for Tbd_2726, *'tesA*, and a kanamycin resistance marker; a schematic illustration of the approach is presented in Fig. 1. After genomic DNA was extracted using the MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI), it was used as the template for six primary PCRs performed using *Taq* DNA polymerase (Qiagen, Venlo, Netherlands; Q-Solution was used for reactions involving *T. denitrificans* genomic DNA):

(i) the *'tesA* gene was amplified from plasmid pJM9 (Müller et al., 2013) by using the forward primer ltesA-f and reverse primer ltesA-r-EcoRI (Table 2) with the following conditions: 94 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 10 min. The amplicon was purified with QIAquick PCR Purification Kit (Qiagen).

**Table 1**
Strains used in this study.

| Strain | Genotype or markers; characteristics and uses | Source or reference |
|---|---|---|
| *Escherichia coli* TOP10 | F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *Φ80lacZΔM15 ΔlacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* ($Str^R$) *endA1 nupG* | Invitrogen |
| *Thiobacillus denitrificans* ATCC 25259 | Wild type | American Type Culture Collection, Manassas, VA. |
| P2425 mutant | ATCC 25259 with Tbd_2545 gene replaced by PCR amplicon containing $P_{2545}$-*'tesA-kan*[a] | This work |
| P2726 mutant | ATCC 25259 with Tbd_2545 gene replaced by PCR amplicon containing $P_{2726}$-*'tesA-kan*[a] | This work |
| Pkan mutant | ATCC 25259 with Tbd_2545 gene replaced by PCR amplicon containing $P_{kan}$-*'tesA-kan* | This work |

[a] $P_{2545}$ and $P_{2726}$ represent promoters included in the upstream regulatory regions of Tbd_2545 (203 bp) and Tbd_2726 (218 bp), respectively.