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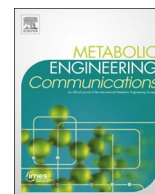
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# Enhanced fatty acid production in engineered chemolithoautotrophic bacteria using reduced sulfur compounds as energy sources



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## ABSTRACT

Chemolithoautotrophic bacteria that oxidize reduced sulfur compounds, such as H<sub>2</sub>S, while fixing CO<sub>2</sub> 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<sub>2</sub>. A modified thioesterase gene from *E. coli* (*tesA*) was integrated into the *T. denitrificans* chromosome under the control of P<sub>kan</sub> 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.

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## 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<sub>2</sub> 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<sub>2</sub> (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<sub>2</sub> 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

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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,  $\beta$ -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 M $\Omega$  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<sub>2</sub> – 10% CO<sub>2</sub> – 5% H<sub>2</sub>. 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<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5H<sub>2</sub>O (20 mM), KNO<sub>3</sub> (20 mM), NH<sub>4</sub>Cl (18.7 mM), KH<sub>2</sub>PO<sub>4</sub> (14.7 mM), NaHCO<sub>3</sub> (30 mM), MgSO<sub>4</sub> · 7H<sub>2</sub>O (3.25 mM), CaCl<sub>2</sub> · 2H<sub>2</sub>O (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  $\mu$ g/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

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

| Primer                      | Sequence <sup>a</sup> (5'–3')            |
|-----------------------------|--|
| ItesA-f                     | ATGGCGGACACGTTATTG                       |
| ItesA-r-EcoRI               | GGGGAATTCCTATGAGTCATGATTAC               |
| Ptbd_2545f-HindIII          | GGGAAGCTTTTCCGTCGGCGTACCGATC             |
| Ptbd_2545r-tesA             | ATCAATAACGTGTCCGCGATGATCTTCCCATCCATCCGAT |
| Ptbd_2726f-HindIII          | GGGAAGCTTTGAGCGCGGATCGAGAAGG             |
| Ptbd_2726r-tesA             | ATCAATAACGTGTCCGCGATTACAGTCTCCGTAGAGGTCG |
| kanP-f-HindIII              | GGGAAGCTTAAAGCCACGTTGTGTCTC              |
| tesA-f-kanP-r               | CAGTAATACAAGGGGTGTTATGGCGGACACGTTATTG    |
| Tbd_2545_KO1-1 <sup>b</sup> | TACTCCTTGTCACGAGGTG                      |
| 2545UP-2-HindIII            | GGGAAGCTTATCGCGGAACCTCTTTTCAT            |
| KO3-EcoRI <sup>b</sup>      | GCAGAATTCACCATCATCGATGAATTG              |
| Tbd_2545_KO6-1 <sup>b</sup> | TCGCGATCGCTACATCGAC                      |

<sup>a</sup> Relevant restriction sites are underlined.

<sup>b</sup> Primer was previously used by Beller et al. (2013).

screw-cap valves (Sigma-Aldrich, St. Louis, MO) under static conditions in an anaerobic glove box.

### 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):

- the 'tesA gene was amplified from plasmid pJM9 (Müller et al., 2013) by using the forward primer ItesA-f and reverse primer ItesA-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                             |
|--|---|---|
| <i>Escherichia coli</i> TOP10                | F <sup>-</sup> mcrA $\Delta$ (mrr-hsdRMS-mcrBC) $\Phi$ 80lacZ $\Delta$ M15 $\Delta$ lacX74 recA1 araD139 $\Delta$ (ara-leu)7697 galU galK | Invitrogen                                      |
| <i>Thiobacillus denitrificans</i> ATCC 25259 | rpsL (Str <sup>R</sup> ) endA1 nupG<br>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 <sup>a</sup>                                       | This work                                       |
| P2726 mutant                                 | ATCC 25259 with Tbd_2545 gene replaced by PCR amplicon containing $P_{2726}$ -tesA-kan <sup>a</sup>                                       | This work                                       |
| Pkan mutant                                  | ATCC 25259 with Tbd_2545 gene replaced by PCR amplicon containing $P_{kan}$ -tesA-kan   | This work                                       |

<sup>a</sup>  $P_{2545}$  and  $P_{2726}$  represent promoters included in the upstream regulatory regions of Tbd\_2545 (203 bp) and Tbd\_2726 (218 bp), respectively.

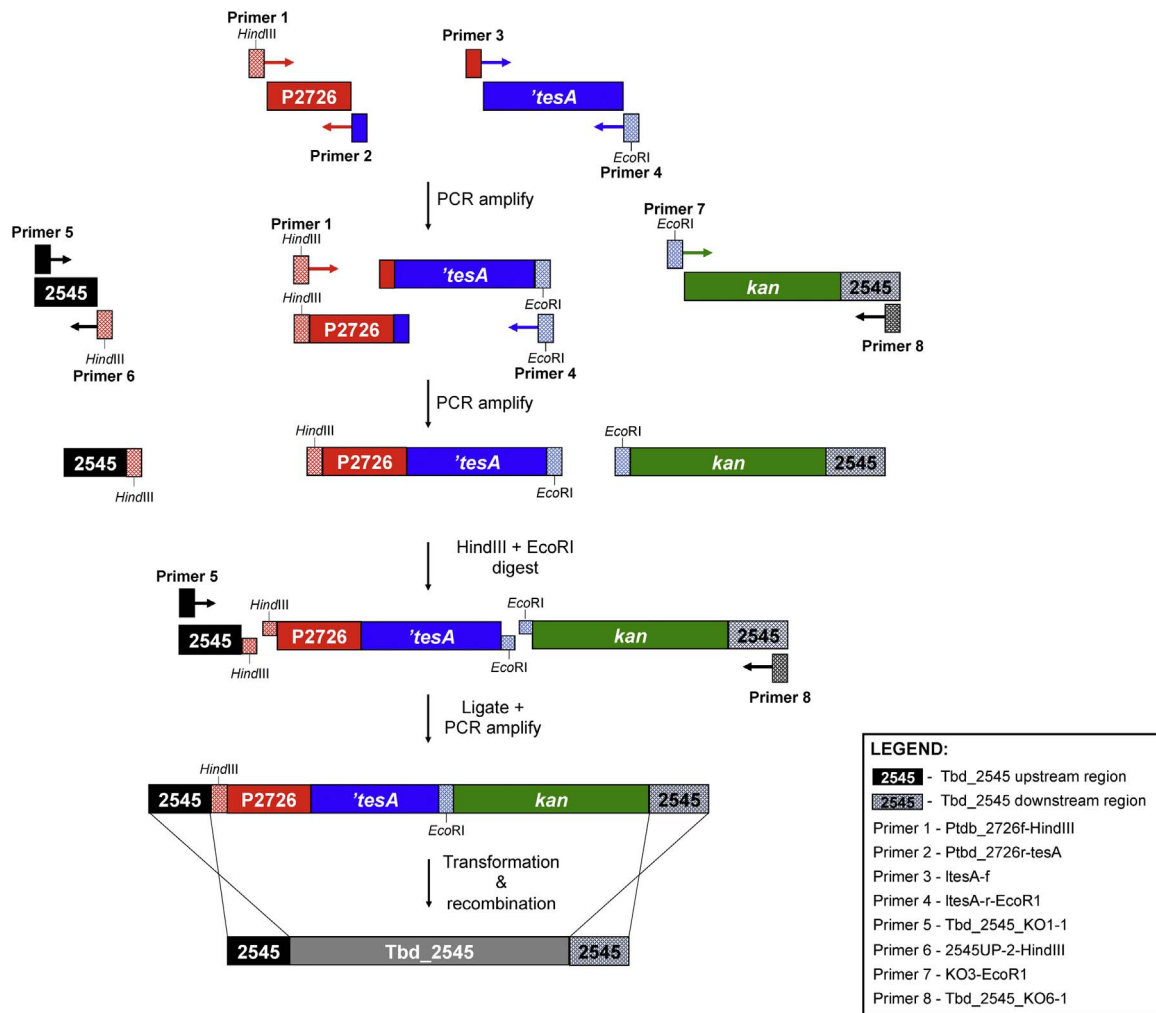


Fig. 1. Schematic illustration of the construction of strain P2726 (Table 1). Primer sequences are given in Table 2.

- (ii) the three promoters were amplified by using three pairs of primers (Ptdb\_2545f-HindIII and Ptdb\_2545r-tesA, Ptdb\_2726f-HindIII and Ptdb\_2726r-tesA, and kanP-f-HindIII and tesA-f-kanP-r) with the same amplification conditions described above.
- (iii) to insert the regulatory region immediately upstream of the ATG site, recombinant PCR (Lloyd et al., 2003) was performed using the mixed, purified PCR products described above. For example, to construct P2726, the primers of Ptdb\_2726f-HindIII and ltesA-r-EcoRI were used in the amplification under 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 1 min and a final extension at 72 °C for 10 min. The amplicon was purified with QIAquick PCR Purification Kit (Qiagen) and digested with HindIII (New England Biolabs, MA), which was introduced with primer Ptdb\_2726f-HindIII, and with EcoRI (New England Biolabs, Ipswich, MA), which was introduced with primer ltesA-r-EcoRI.
- (iv) primers Tbd\_2545\_KO1-1 (Beller et al., 2013) and 2545UP-2-HindIII (Table 2) were used to amplify the upstream sequence of the Tbd\_2545 gene using the same conditions as described in (i). The amplicon was purified with QIAquick PCR Purification Kit (Qiagen) and digested with HindIII (New England Biolabs), which was introduced with primer 2545UP-2-HindIII.
- (v) primers KO3-EcoRI and Tbd\_2545\_KO6-1 (Table 2) were used to amplify the kanamycin resistance cassette and downstream sequence of the Tbd\_2545 gene using the genomic DNA of Tbd\_2545 mutant as a template (Beller et al., 2013) (with the same PCR conditions listed above, except the 72 °C cycles were 2 min) and the amplicon was purified with QIAquick PCR Purification Kit (Qiagen) and was digested with EcoRI (New England Biolabs), which was introduced with primer KO3-EcoRI;
- (vi) The three digested PCR products from (iii), (iv), and (v) were ligated using a Fast-Link DNA Ligation Kit (Epicentre Biotechnologies). The ligation product was used as a template for recombinant PCR, which was carried out with primers Tbd\_2545\_KO1-1 and Tbd\_2545\_KO6-1 using 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 3 min and a final extension at 72 °C for 10 min. The recombinant PCR product was purified and used for transformation of *T. denitrificans*, which was performed with ~100 ng of DNA according to methods described previously (Letain et al., 2007). After electroporation, cells were spread on agar plates containing 50 µg/mL kanamycin and cultured under denitrifying conditions at 30 °C in an anaerobic glove box. Mutant colonies were cultured with growth medium containing 50 µg/mL kanamycin. The gene replacement was confirmed by PCR with primers Tbd\_2545\_KO1-1 and Tbd\_2545\_KO6-1 using the genomic DNA from the mutant.

## 2.4. Analytical methods

Sulfate, nitrate, nitrite, and chloride concentrations were measured by ion chromatography (IC) using a Model ICS-2000 IC (Dionex Corporation, Sunnyvale, CA) with micromembrane suppression and electrochemical conductivity detection, as described previously (Han et al., 2010). Quantification relied on external standards using a 5-point calibration.

### 2.4.1. Extraction, derivatization, and GC/MS analysis of fatty acids

Two hundred-milliliter *T. denitrificans* cultures were harvested by centrifugation within 5 days of depleting available nitrate ( $13,400 \times g$ , 4 °C, 15 min), the pellet from each strain was re-suspended in 2 mL of phosphate-buffered saline solution, and the suspended cells were transferred to acetone-rinsed 30-mL glass tubes with PTFE-lined screw-cap closures. Cells were centrifuged at  $2500 \times g$  for 15 min (20 °C) and the aqueous phase was decanted. Extraction and GC/MS analysis were then conducted based on methods described previously (Beller et al., 2010). The pellet was amended with 100  $\mu$ L of reagent water and the mixture was homogenized with a vortex mixer. Then 1 mL of high-purity methanol (B&J Brand, Honeywell, Muskegon, MI;  $\geq 99.9\%$  purity) and 4 mL of high-purity hexane were added to the cells; the hexane was amended with perdeuterated alkane internal standards ( $C_{24}D_{50}$  and  $C_{10}D_{22}$ ) to assess sample-specific analytical recovery. The cell-solvent mixture was homogenized with a vortex mixer and sonicated in an ice bath for 15 min, allowed to sit for 10 min, and then centrifuged at  $2500 \times g$  for 15 min (20 °C). The hexane layer was then removed with a solvent-cleaned Pasteur pipette and transferred to a glass, 10-mL conical vial. The hexane was concentrated under a gentle stream of ultra high-purity  $N_2$ , derivatized with ethereal diazomethane (Beller et al., 2010), and then concentrated to 100  $\mu$ L for GC/MS analysis. Organic solvents contacted only glass or PTFE, and all glass and PTFE surfaces were rigorously pre-cleaned with high-purity acetone.

Electron ionization GC/MS analyses were performed with a model 7890 A GC (Agilent, Palo Alto, CA) with an HP-5MS fused silica capillary column (30-m length, 0.25-mm inner diameter, 0.25- $\mu$ m film thickness; Agilent) coupled to an HP 5975 C series mass selective detector; 1  $\mu$ L injections were performed by a model 7683B autosampler. The GC oven was programmed from 40 °C (held for 3 min) to 300 °C at 15 °C/min and then held for 5 min; the injection port temperature was 250 °C, and the transfer line temperature was 280 °C. The carrier gas, ultra high-purity helium, flowed at a constant rate of 1 mL/min. Injections were splitless, with the split turned on after 0.5 min. Data acquisition was in full-scan mode, with the MS scanning from  $m/z$  50 to 600 at a rate of 2.7 scans per s. External standard quantification was performed using three-point calibration curves with methyl hexadecanoate standards.

## 3. Results and discussion

All strains consumed from 75% to 100% of the nitrate in the

**Table 3**  
Nitrate consumption and sulfate production during incubation.

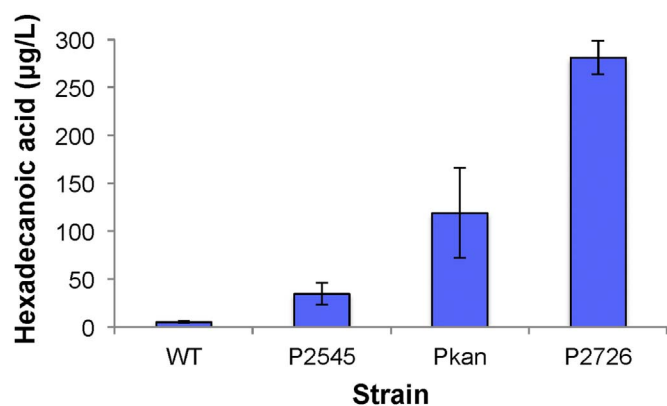
| Strain    | Initial Nitrate (mM) | Final Nitrate (mM) | Initial Sulfate (mM) | Final Sulfate (mM) | Sulfate produced/nitrate consumed | > 75% nitrate removal (hr) |
|-----------|----------------------|--------------------|----------------------|--------------------|-----------------------------------|----------------------------|
| Wild type | 21 $\pm$ 1.1         | 0 $\pm$ 0          | 4.6 $\pm$ 0.7        | 33 $\pm$ 0.3       | 1.36                              | 48                         |
| P2545     | 19 $\pm$ 1.9         | 0 $\pm$ 0          | 4.6 $\pm$ 0.7        | 33 $\pm$ 0.3       | 1.46                              | 240                        |
| P2726     | 22 $\pm$ 5.0         | 4.7 $\pm$ 0.8      | 4.3 $\pm$ 0.1        | 24 $\pm$ 2.4       | 1.14                              | 215                        |
| Pkan      | 24 $\pm$ 1.3         | 1.6 $\pm$ 2.3      | 4.0 $\pm$ 0.04       | 30 $\pm$ 5.4       | 1.16                              | 305                        |

growth medium during incubation and correspondingly oxidized an amount of thiosulfate to sulfate consistent with stoichiometric expectations: observed molar ratios of sulfate produced per nitrate consumed were typically within 10% of the expected ratio of 1.25 (Table 3). However, rates of metabolism varied among the strains: the wild type consumed all nitrate within 48 h while the engineered strains took longer (Table 3). Notably, there was no positive or negative correlation between metabolic rate and fatty acid production among the samples, as indicated below.

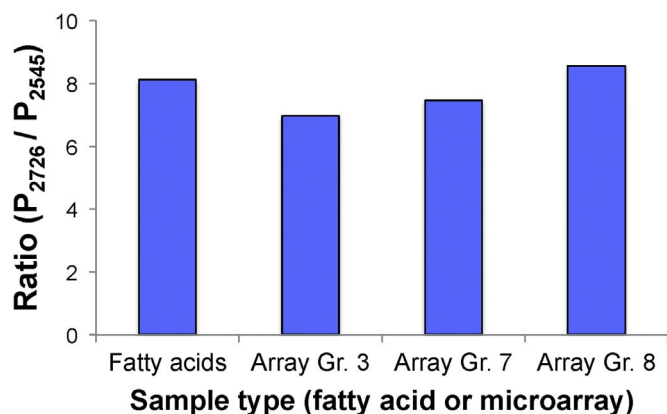
Fatty acid production varied widely among the *T. denitrificans* strains, spanning 1.7 orders of magnitude (Fig. 2). Regardless of the upstream promoter, chromosomal integration of *tesA* resulted in marked increases in fatty acid production (Fig. 2). Ratios of titers of hexadecanoic acid, the dominant fatty acid in this species (Agate and Vishniac, 1973 and unpublished data), in engineered strains relative to titers in the wild-type ranged from 6.4 (P2545) to 22 (Pkan) to 52 (P2726).

For the two native promoters, it is instructive to compare their apparent relative strength based on fatty acid production to their relative strength based on expression of their cognate genes (i.e., genes *Tbd\_2545* and *Tbd\_2726* correspond to  $P_{2545}$  and  $P_{2726}$ , respectively). Indications of the relative strength of native promoters in *T. denitrificans* ATCC 25259 were presented in a previous whole-genome transcriptional study of this strain (Beller et al., 2013). In that study, there were three sets of samples (microarray Groups 3, 7, and 8) that were cultivated under conditions comparable to those used in the present study, namely, thiosulfate oxidation under denitrifying conditions at 30 °C. In Fig. 3, we have compared the relative expression of genes *Tbd\_2726* and *Tbd\_2545* in those microarray samples with the ratio of hexadecanoic acid produced in engineered strains with the promoter for *Tbd\_2726* vs. the promoter for *Tbd\_2545*. The results are strikingly similar, suggesting that there is a strong relationship between promoter strength, *tesA* expression, and fatty acid production.

This proof-of-principle study demonstrated that it should be possible to, in a predictable fashion, substantially enhance fatty acid titers in a chemolithoautotrophic bacterium that could remediate sulfide-laden wastewater and divert fixed  $CO_2$  to useful and renewable products. Although fatty acid titers were relatively low in this study, several ameliorating factors should be borne in mind: (i) many other engineering modifications could potentially be made to further enhance fatty acid production, such as up-regulating acetyl-CoA carboxylase, (ii) this technology is not solely intended for producing renewable compounds from an effectively cost-free feedstock (sulfide in wastewater) – it is also intended for cost-effective remediation of sulfide-laden wastewater, which could replace relatively expensive chemical treatment that is commonly used, (iii) it could be used to produce value-added, fatty acid-derived compounds, such as medium-chain methyl ketones (valued at up to \$1000/kg; Beller et al., 2015), which would have lower production requirements for commercial viability, (iv) it might result in much higher titers when coupled to pathways that provide a strong metabolic pull. Regarding the last point, an example exists of engineered bacteria that had very low fatty acid titers after use of *tesA* but much higher titers of methyl ketones



**Fig. 2.** Hexadecanoic acid production in engineered *T. denitrificans* strains expressing the *tesA* thioesterase gene under control of various promoters (see Table 1 for details). Error bars represent one standard deviation.



**Fig. 3.** Relative strength of two native *T. denitrificans* promoters, P<sub>2726</sub> and P<sub>2545</sub>, as measured by fatty acid titer for strains P2726 and P2545, which use these promoters to drive *tesA* expression (Table 1), or expression of their cognate genes in transcriptional studies. See text for more detail on the microarray studies (Array Groups 3, 7, and 8).

when that metabolic capability was added. Specifically, *Ralstonia eutropha* H16 had a fatty acid titer of only 17 µg/L when over-expressing *tesA* (more than an order lower than observed for *T. denitrificans* strain P2726), but after a methyl ketone production pathway was added to this strain, it had a methyl ketone titer that was three to four orders of magnitude higher than the titer of the methyl ketone precursors, fatty acids, in the background strain without the methyl ketone pathway (Müller et al., 2013).

Future studies will explore methods to further increase fatty acid titers in engineered strains of *T. denitrificans* and addition of pathways to convert fatty acids to more valuable products, such as medium-chain methyl ketones.

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## References

- Agate, A.D., Vishniac, W., 1973. Characterization of *Thiobacillus* species by gas-liquid chromatography of cellular fatty acids. *Arch. Mikrobiol.* 89, 257–267.
- Beller, H.R., Chain, P.S.G., Letain, T.E., Chakicherla, A., Larimer, F.W., Richardson, P.M., Coleman, M.A., Wood, A.P., Kelly, D.P., 2006. The genome sequence of the obligately chemolithoautotrophic, facultatively anaerobic bacterium *Thiobacillus denitrificans*. *J. Bacteriol.* 188, 1473–1488.
- Beller, H.R., Goh, E.B., Keasling, J.D., 2010. Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*. *Appl. Environ. Microbiol.* 76, 1212–1223.
- Beller, H.R., Lee, T.S., Katz, L., 2015. Natural products as biofuels and bio-based chemicals: fatty acids and isoprenoids. *Nat. Prod. Rep.* 32, 1508–1526.
- Beller, H.R., Legler, T.C., Kane, S.R., 2012. Genetic manipulation of the obligate chemolithoautotrophic bacterium *Thiobacillus denitrificans*. In: Navid, A. (Ed.), *Microbial Systems Biology: Methods and Protocols* vol. 881. Springer Science, New York, NY, pp. 99–136.
- Beller, H.R., Zhou, P., Legler, T.C., Chakicherla, A., Kane, S., Letain, T.E., O'Day, P.A., 2013. Genome-enabled studies of anaerobic, nitrate-dependent iron oxidation in the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Front. Microbiol.* 4, 249.
- Cardoso, R.B., Sierra-Alvarez, R., Rowlette, P., Flores, E.R., Gomez, J., Field, J.A., 2006. Sulfide oxidation under chemolithoautotrophic denitrifying conditions. *Biotechnol. Bioeng.* 95, 1148–1157.
- Cho, H., Cronan Jr., J.E., 1995. Defective export of a periplasmic enzyme disrupts regulation of fatty acid synthesis. *J. Biol. Chem.* 270, 4216–4219.
- Garcia-de-Lomas, J., Corzo, A., Carmen Portillo, M., Gonzalez, J.M., Andrades, J.A., Saiz-jimenez, C., Garcia-Robledo, E., 2007. Nitrate stimulation of indigenous nitrate-reducing, sulfide-oxidising bacterial community in wastewater anaerobic biofilms. *Water Res.* 41, 3121–3131.
- Han, R., Geller, J.T., Yang, L., Brodie, E.L., Chakraborty, R., Larsen, J.T., Beller, H.R., 2010. Physiological and transcriptional studies of Cr(VI) reduction under aerobic and denitrifying conditions by an aquifer-derived pseudomonad. *Environ. Sci. Technol.* 44, 7491–7497.
- Kleerebezem, R., Mendez, R., 2002. Autotrophic denitrification for combined hydrogen sulfide removal from biogas and post-denitrification. *Water Sci. Technol.* 45, 349–356.
- Letain, T.E., Kane, S.R., Legler, T.C., Salazar, E.P., Agron, P.G., Beller, H.R., 2007. Development of a genetic system for the chemolithoautotrophic bacterium *Thiobacillus denitrificans*. *Appl. Environ. Microbiol.* 73, 3265–3271.
- Lloyd, J.R., Leang, C., Hodges Myerson, A.L., Coppi, M.V., Cuifo, S., Methe, B., Sandler, S.J., Lovley, D.R., 2003. Biochemical and genetic characterization of PpcA, a periplasmic c-type cytochrome in *Geobacter sulfurreducens*. *Biochem. J.* 369, 153–161.
- Manconi, I., Carucci, A., Lens, P., 2007. Combined removal of sulfur compounds and nitrate by autotrophic denitrification in bioaugmented activated sludge system. *Biotechnol. Bioeng.* 98, 551–560.
- Müller, J., MacEachran, D., Burd, H., Sathitsuksanoh, N., Bi, C., Yeh, Y.C., Lee, T.S., Hillson, N.J., Chhabra, S.R., Singer, S.W., Beller, H.R., 2013. Engineering of *Ralstonia eutropha* H16 for autotrophic and heterotrophic production of methyl ketones. *Appl. Environ. Microbiol.* 79, 4433–4439.
- Ongcharit, C., Sublette, K.L., Shah, Y.T., 1991. Oxidation of hydrogen sulfide by flocculated *Thiobacillus denitrificans* in a continuous culture. *Biotechnol. Bioeng.* 37, 497–504.
- Shao, M.F., Zhang, T., Fang, H.H., 2010. Sulfur-driven autotrophic denitrification: diversity, biochemistry, and engineering applications. *Appl. Microbiol. Biotechnol.* 88, 1027–1042.
- Steen, E.J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S.B., Keasling, J.D., 2010. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562.
- Sublette, K.L., Sylvester, N.D., 1987. Oxidation of hydrogen sulfide by continuous cultures of *Thiobacillus denitrificans*. *Biotechnol. Bioeng.* 29, 753–758.
- Widdel, F., Bak, F., 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows, A., Dworkin, H.G.T., M., Harder, W., Schleifer, K.H. (Eds.), *The Prokaryotes* vol. 4. Springer-Verlag, New York, N.Y., pp. 3352–3370.