Biodegradation (2009) 20:45–53
DOI 10.1007/s10532-008-9198-y

ORIGINAL PAPER

# Identification of *c*-type cytochromes involved in anaerobic, bacterial U(IV) oxidation

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Received: 29 February 2008 / Accepted: 29 April 2008 / Published online: 11 May 2008


**Abstract** Anaerobic, bacterial reduction of water-soluble U(VI) complexes to the poorly soluble U(IV) mineral uraninite has been intensively studied as a strategy for in situ remediation of uranium-contaminated groundwater. A novel and potentially counteracting metabolic process, anaerobic, nitrate-dependent U(IV) oxidation, has recently been described in two bacterial species (*Geobacter metallireducens* and *Thiobacillus denitrificans*), but the underlying biochemistry and genetics are completely unknown. We report here that two diheme, *c*-type cytochromes (putatively $c_4$ and $c_5$ cytochromes) play a major role in nitrate-dependent U(IV) oxidation by *T. denitrificans*. Insertion mutations in each of the two genes encoding these cytochromes resulted in a greater than 50% decrease in U(IV) oxidation activity, and complementation in trans restored activity to wild-type levels. Sucrose-density-gradient ultracentrifugation confirmed that both cytochromes are membrane-associated. Insertion mutations in genes encoding other membrane-associated, *c*-type cytochromes did not diminish U(IV) oxidation. This is the first report of proteins involved in anaerobic U(IV) oxidation.

**Keywords** Cytochrome · Anaerobic · *Thiobacillus denitrificans* · Uranium · Genetic system

**Electronic supplementary material** The online version of this article (doi:10.1007/s10532-008-9198-y) contains supplementary material, which is available to authorized users.

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## Introduction

The remediation of uranium-contaminated groundwater and soil is a major environmental concern of the U.S. Department of Energy (DOE), whose Cold War activities were responsible for 1.7 trillion gallons of contaminated groundwater in 5,700 distinct plumes; more than 60% of DOE facilities include radionuclides (predominantly uranium) as a contaminant class (U.S. Department of Energy 2003). In situ reductive immobilization of uranium, whereby indigenous, anaerobic bacteria can reduce soluble uranyl complexes to poorly soluble uraninite ($UO_2$), has undergone intensive study since its proposal in the early 1990s and has been the subject of hundreds of scientific articles. Anaerobic, nitrate-dependent U(IV) oxidation, first reported in 2002 (Finneran et al. 2002; Senko et al. 2002), could complicate efforts at long-term reductive immobilization, as nitrate is a common co-contaminant with uranium at

DOE sites. Beyond the environmental relevance of nitrate-dependent U(IV) oxidation, it represents an intriguing bacterial metabolic capability about which nothing is known at a molecular level.

The two bacteria currently documented to catalyze nitrate-dependent U(IV) oxidation, *Geobacter metallireducens* (Finneran et al. 2002) and *Thiobacillus denitrificans* (Beller 2005), have very different lifestyles. *G. metallireducens* is a chemoorganoheterotroph, whereas *T. denitrificans* is an obligate chemolithoautroph best known for its ability to conserve energy from the oxidation of inorganic sulfur compounds under either aerobic or denitrifying conditions at circumneutral pH (Beller et al. 2006a). Among the sulfur-containing electron donors used by *T. denitrificans* are minerals such as FeS and pyrite ($FeS_2$).

Uraninite presents unusual challenges to bacteria as an electron donor by virtue of its low solubility [which would tend to impede U(IV) transport across the outer membrane and consequently would disfavor intracellular oxidation] and relatively high midpoint reduction potential. The reduction potential ($E_0'$) of the $UO_2^{2+}/UO_2(s)$ couple is 410 mV or 260 mV for crystalline or amorphous $UO_2$, respectively (Beller 2005). In light of the thermodynamic and physicochemical constraints that $UO_2$ oxidation imposes on redox proteins, we set out to identify proteins in *T. denitrificans* that could potentially satisfy those constraints, namely, outer-membrane (OM), *c*-type cytochromes. In this paper, we describe efforts to identify OM, *c*-type cytochromes expressed in *T. denitrificans* under denitrifying conditions, construction of transposon insertion mutants for the genes encoding those cytochromes, and use of in vivo assays to determine whether any of those mutants were defective in anaerobic, nitrate-dependent U(IV) oxidation.

## Materials and methods

### Bacterial strains and growth conditions

The strains and plasmids used in this study are listed and described in Table 1. *Escherichia coli* was grown in Luria–Bertani broth or Terrific Broth at 37°C with shaking. *Thiobacillus denitrificans* (ATCC 25259) was cultured using anaerobic conditions and medium described previously (Beller 2005; Beller et al. 2006a; Letain et al. 2007).

### DNA manipulations

Genomic DNA was isolated from *T. denitrificans* using a MasterPure Total Nucleic Acid Isolation kit (EpiCentre, Madison, WI). Plasmid DNA was isolated from *E. coli* and *T. denitrificans* using midi- or mini-plasmid purification kits (QIAGEN, Valencia, CA). PCR products were purified with a QIAquick gel extraction kit (QIAGEN). Primers for amplification of *T. denitrificans* genomic DNA were designed from the whole-genome sequence (Beller et al. 2006a). Advantage-GC 2 polymerase was purchased from Clontech (Mountain View, CA) and used for all PCR amplifications except where noted. Restriction endonucleases and ligase were purchased from New England Biolabs (Ipswich, MA).

### Construction of transposon insertion mutants and complementation vector

*T. denitrificans* insertion mutants were created using methods described previously (Letain et al. 2007). PCR primers (Supplemental Table 1) were designed with KpnI restriction sites and bracketed regions ca. 1 kb upstream and downstream of the target genes. PCR conditions (98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 64°C for 30 s, 72°C for 4 min, and a final extension of 8 min) were the same for all reactions except for Tbd_0723, which had an extension time of 5 min. Cloning, in vitro transposition, sequencing, competent cell preparation, electroporation, and screening of insertion mutants were all performed as previously described (Letain et al. 2007), with the exception that linear DNA for electroporation into *T. denitrificans* was generated using primers described in Supplemental Table 1, with the following PCR conditions: 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 5 min, and a final extension of 8 min. The reaction for Tbd_0723 had an extension time of 6 min.

The complementation plasmid pTL2 (Table 1) was used to create the Tbd_0187 expression vector, pTL4, using methods described previously (Letain et al. 2007). Primers Tbd0187ATG-f,r (Supplemental Table 1) were used to generate the 628-bp amplicon containing Tbd_0187, which was subsequently

**Table 1** Strains and plasmids used

| Strain, plasmid, or transposon | Genotype or markers; characteristics and uses | Source or reference |
|---|---|---|
| Strains | | |
| *Escherichia coli* | F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *ϕ*80*lacZ*Δ*M15* Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7967 galU galK rpsL* (Str$^R$) *endA1 nupG* | Invitrogen |
| *Thiobacillus denitrificans* | | |
| ATCC 25259 | Wild-type | ATCC |
| Tbd_1948 mutant | Tbd_1948::kan | This work |
| Tbd_2726 mutant | Tbd_2726::kan | This work |
| Tbd_0187 mutant | Tbd_0187::kan | This work |
| Tbd_0146 mutant | Tbd_0146::kan | This work |
| Tbd_0723 mutant | Tbd_0723::kan | This work |
| Tbd_1398 mutant | Tbd_1398::kan | This work |
| Plasmids | | |
| pUC19 | pMB1, Amp$^R$, cloning vector | |
| pTL2 | IncP, Gent$^R$, Amp$^S$, pTL1 with *bla*::gent; *T. denitrificans* expression vector | (Letain et al. 2007) |
| pTL4 | IncP, Gent$^R$, pTL2 with Tbd_0187 coding sequence inserted next to $P_{Kan}$ allowing for expression | This work |
| pUC19-Tbd1948 | pMB1, Amp$^R$, pUC19 with Tbd_1948 (−1778, +2041) inserted at MCS[a] | This work |
| pUC19-Tbd1948::kan | pUC19-Tbd1948 with Tn-kan inserted at +217 of Tbd_1948 | This work |
| pUC19-Tbd2726 | pMB1, Amp$^R$, pUC19 with Tbd_2726 (−1830, +1986) inserted at MCS[a] | This work |
| pUC19-Tbd2726::kan | pUC19-Tbd2726 with Tn-kan inserted at +230 of Tbd_2726 | This work |
| pUC19-Tbd0187 | pMB1, Amp$^R$, pUC19 with Tbd_0187 (−867, +1396) inserted at MCS[a] | This work |
| pUC19-Tbd0187::kan | pUC19-Tbd0187 with Tn-kan inserted at +73 of Tbd_0187 | This work |
| pUC19-Tbd0146 | pMB1, Amp$^R$, pUC19 with Tbd_0146 (−724, +1262) inserted at MCS[a] | This work |
| pUC19-Tbd0146::kan | pUC19-Tbd0146 with Tn-kan inserted at +33 of Tbd_0146 | This work |
| pUC19-Tbd0723 | pMB1, Amp$^R$, pUC19 with Tbd_0723 (−733, +2677) inserted at MCS[a] | This work |
| pUC19-Tbd0723::kan | pUC19-Tbd0723 with Tn-kan inserted at +126 of Tbd_0723 | This work |
| pUC19-Tbd1398 | pMB1, Amp$^R$, pUC19 with Tbd_1398 (−1066, +1322) inserted at MCS[a] | This work |
| pUC19-Tbd1398::kan | pUC19-Tbd1398 with Tn-kan inserted at +137 of Tbd_1398 | This work |
| Transposons | | |
| Tn-kan | Kan$^R$, EZ-Tn5 <KAN-2> DNA fragment with kanamycin resistance selection marker located between Mosaic End (ME) Tn*5* transposase recognition sequences | Epicentre |

[a] pUC19 variants are named using "Tbd" locus tags (GenBank accession no. CP000116) for coding sequences (CDS) of interest and contain flanking genomic sequence. Flanking sequence is defined by numbers in parenthesis, where A of the ATG start site of the relevant CDS is designated +1

ligated into the unique HpaI site of pTL2. The resulting clones were screened by PCR analysis for correct orientation of Tbd_0187 relative to $P_{Kan}$ and the sequence was confirmed.

## Sucrose-density-gradient ultracentrifugation

Cell-free extracts for *T. denitrificans* cells induced under denitrifying, thiosulfate- or $FeCO_3$ (siderite)-oxidizing conditions (Beller 2005; Beller et al. 2006a, b) were generated by harvesting cells anaerobically by centrifugation, resuspending the cells in 10 mM HEPES buffer, and passing them through a French pressure cell three times (at 138 MPa). Cells induced under $FeCO_3$-oxidizing, denitrifying conditions were first washed (10 min, 4°C) with a 10 mM HEPES buffer containing 5 mM EDTA before resuspension in 10 mM HEPES; this step was

included to dissolve Fe-containing precipitates. Cell-free extracts were subjected to sucrose-density-gradient fractionation as previously described (Larsen et al. 1999). Briefly, extracts containing 10–20 mg protein were loaded onto 30–55% (w/w) sucrose gradients and resolved in a Beckman SW41 rotor (Beckman model L8-70 ultracentrifuge) at 39,000 rpm (4°C) for 24 h. Fractions were recovered by pumping 60% (w/w) sucrose into the centrifuge tube bottom and collecting 0.5-ml aliquots at the tube top with a Tube Piercer apparatus (Teledyne Isco, Inc.).

### One-dimensional (1-D) gel electrophoresis, heme protein visualization, and de novo peptide sequencing

Proteins subjected to electrophoretic separation were first precipitated with trichloroacetic acid. Samples intended for 1-D analysis were resuspended in 1× LDS sample buffer (Invitrogen, Carlsbad, CA). Protein samples were separated on NuPAGE Novex 4-12% Bis-Tris mini gels with 1× NuPAGE MES SDS running buffer (Invitrogen) for 40 min at 200 V. No reducing agents were used and proteins were not heat-denatured. Following 1-D electrophoresis, proteins were transferred to Invitrolon polyvinylidene fluoride (PVDF) membranes (Invitrogen) in 1× Transfer buffer (Invitrogen)/20% methanol at 100 V for 1 h. Heme-containing proteins were visualized by luminol-based chemiluminescence as described elsewhere (Dorward 1993) (ECL Western Blotting Detection Reagents, GE Healthcare). PVDF membranes were post-stained with 0.1% Coomassie Brilliant Blue/50% methanol/10% acetic acid. Duplicate 1-D gel electrophoresis samples were separated simultaneously (in lanes that were not blotted) and bands corresponding to those identified by heme visualization were excised. Excised bands were subjected to de novo peptide sequencing, which was performed by ProtTech, Inc. (Norristown, PA) using nano-liquid chromatography-tandem mass spectrometry (LC/MS/MS) with a quadrupole ion trap instrument (Thermo, Palo Alto, CA). Briefly, each protein gel band of interest was destained, cleaned, and digested in-gel with sequencing grade modified trypsin. The mass spectrometric data acquired were used to search the most recent non-redundant protein database (GenBank).

### In vivo U(IV) oxidation assay

Cell suspension assays of anaerobic, nitrate-dependent uraninite oxidation were performed as described elsewhere, including positive and negative live controls (Beller 2005).

## Results and discussion

### Identification of membrane-associated *c*-type cytochromes in *T. denitrificans*

We performed sucrose-density-gradient ultracentrifugation separations on extracts of *T. denitrificans* cells that had been induced under denitrifying conditions with either thiosulfate or the Fe(II)-containing mineral siderite ($FeCO_3$) as the electron donor. Several lines of evidence, including positive lipopolysaccharide silver staining results for SDS-PAGE gels (Tsai and Frasch 1982) and migration to a specific gravity range (1.1785–1.185) consistent with OM proteins in some other bacteria (Huntley et al. 2007), led us to conclude that two fractions were enriched in OM proteins. These two fractions were combined, the associated proteins were separated by SDS-PAGE, and the heme proteins (*c*-type cytochromes) were visualized using a luminol-based chemiluminescence assay (Fig. 1; Lanes 1 and 2). Under both induction conditions tested, six bands containing heme proteins were visible (albeit with different levels of relative expression) (bands A–F, Fig. 1). De novo peptide sequencing of tryptic digests of these six bands using nano-LC/MS/MS revealed that the following *c*-type cytochromes were included in these bands: A—Tbd_0077 (NirS), B—Tbd_0723, C—Tbd_0146, D—Tbd_1398, E—Tbd_0187, and F—Tbd_0562 (NorC) (see peptide sequences in Supplementary Table 2); Tbd locus tags correspond to the genome sequence (Beller et al. 2006a) in GenBank (accession number CP00016). These are not all OM proteins, as NorC (a nitric oxide reductase subunit) is an integral cytoplasmic membrane (CM) protein with one transmembrane domain and NirS (nitrite reductase) is a periplasmic protein that has also been found to be membrane-associated in another *T. denitrificans* strain (Hole et al. 1996). Nonetheless, Coomassie staining

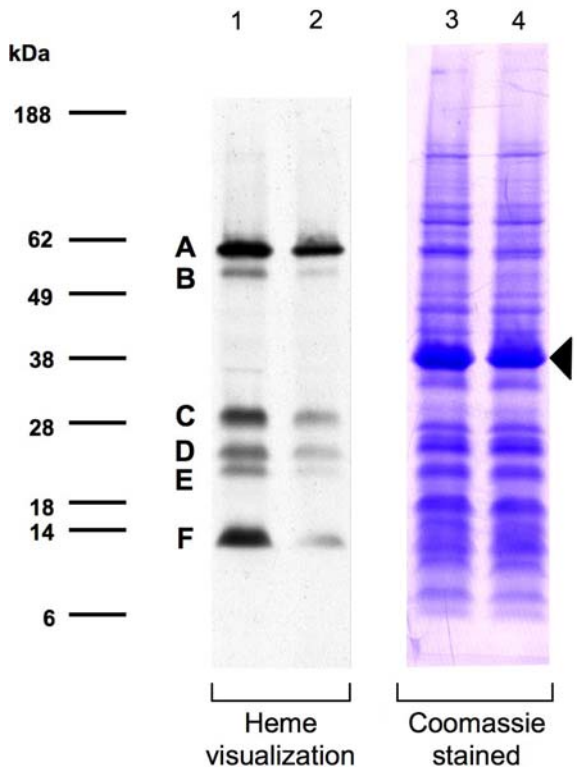


**Fig. 1** Profiles of heme proteins and total proteins in the putative outer-membrane fraction of *T. denitrificans* expressed under denitrifying conditions. Two fractions (specific gravity = 1.1785–1.185) from sucrose-density-gradient ultracentrifugation were combined and separated by 1-D gel electrophoresis. Heme proteins (Lanes 1 and 2; 40 μg total protein per lane) were visualized after transfer to a PVDF membrane and assayed with a luminol-based chemiluminescence technique. Lanes 3 and 4 represent the Coomassie-stained membrane after heme visualization. Lane 1—heme proteins, $FeCO_3$-induced cells (bands A–F are addressed in the text); Lane 2—heme proteins, thiosulfate-induced cells; Lane 3—Coomassie-stained proteins, $FeCO_3$-induced cells; Lane 4—Coomassie-stained proteins, thiosulfate-induced cells. Molecular masses are denoted in kDa. The arrow in Lane 4 indicates an outer-membrane porin (see text)

of the same blot (Lanes 3 and 4, which correspond respectively to Lanes 1 and 2), subsequent 2-D electrophoretic separations, and de novo peptide sequencing of the dominant spot at ~38 kDa revealed that an OM porin (Tbd_0448; indicated by an arrow in Fig. 1) is clearly the most abundant protein in the combined fractions. Regardless of the incomplete resolution of OM and CM proteins in this separation process, the proteins in the characterized fractions are all membrane-associated.

## Construction of transposon insertion mutants for membrane-associated *c*-type cytochromes

Using a genetic system that we recently developed for *T. denitrificans* (Letain et al. 2007), we constructed kan transposon insertion mutants for the membrane-associated, *c*-type cytochromes represented by bands B–E in Fig. 1. Mutants were not made for *nirS* or *norC* (bands A and F) both because their functions are well known (unlike the other four genes) and because the mutants would probably not be viable since the gene products are essential to denitrification. Mutants were made for two additional genes based on whole-genome transcriptional data for *T. denitrificans*. Tbd_2726 was targeted because it is a constitutively and very highly expressed *c*-type cytochrome of unknown function (Beller et al. 2006b) (GEO accession number GSE5256). Tbd_1948 was targeted because it was highly upregulated (9- to 39-fold) under various nitrate-dependent, Fe(II)- and U(IV)-oxidizing conditions relative to nitrate-dependent, thiosulfate-oxidizing conditions.

## Identification of *c*-type cytochrome mutants defective in nitrate-dependent U(IV) oxidation

The six mutants and wild-type *T. denitrificans* were assayed for their ability to catalyze nitrate-dependent U(IV) oxidation under strictly anaerobic conditions. The cell suspension assays were conducted as described previously (Beller 2005) and included live negative controls (identical to active samples except that they lacked nitrate) and positive controls (to confirm that the cells were healthy by assessing the specific denitrification rate with thiosulfate as the electron donor). The results, summarized in Table 2, clearly demonstrate that the mutants of Tbd_0146 and Tbd_0187 were markedly (more than 50%) defective in U(IV) oxidation as compared to the wild-type. To demonstrate that the insertion mutation was responsible for the observed effect, we complemented the Tbd_0187 mutant in trans (Letain et al. 2007) and observed completely restored activity. PCR analyses confirmed that the mutants and complemented Tbd_0187 mutant were of the expected genotype (Fig. 2). While the Tbd_0146 and Tbd_0187 mutants were clearly defective, their residual U(IV) oxidation activity still showed a strong dependence on the presence of nitrate. In fact, the

**Table 2** Summary of anaerobic U(IV) oxidation experiments with *T. denitrificans* strains

| Strain | U(IV) oxidation[a] (% of wild-type; mean ± s.d.) | Specific denitrification activity (positive control) (nmol electron equivalents $min^{-1}$ mg $protein^{-1}$)[b] | Protein (mg) |
|---|---|---|---|
| Wild-type | 100 | 4700 | 0.67 |
| Tbd_0146 mutant | 48 ± 0.3 | 2900 | 0.84 |
| Tbd_0187 mutant | 47 ± 12 | 3000 | 0.64 |
| Complemented Tbd_0187 mutant | 130 ± 9.2[c] | 2600 | 0.61 |
| Tbd_0723 mutant | 120 ± 13 | 3000 | 1.16 |
| Tbd_1398 mutant | 76 ± 7.1[c,d] | 4000 | 0.90 |
| Tbd_2726 mutant | 110 ± 9.9 | 2500 | 0.30 |
| Tbd_1948 mutant | 130 ± 6.5 | 3100 | 1.41 |

[a] Calculated as the difference between soluble U in the presence of nitrate minus soluble U in the absence of nitrate at the termination of the 7-day cell suspension experiment, expressed as a percent of the wild-type value (thus, the wild-type is 100 by definition). The mean ± one standard deviation is given for triplicate suspensions unless indicated otherwise

[b] Positive controls were performed with thiosulfate as the electron donor as described elsewhere (Beller 2005)

[c] Duplicate rather than triplicate suspensions were performed

[d] This mutant was assayed with a different batch of $UO_2$ than was used for all other experiments reported here. Studies with wild-type *T. denitrificans* demonstrated that this batch was more refractory to oxidation than the batch used for all other experiments. We have accounted for this by normalizing the U(IV) oxidation results for the Tbd_1398 mutant to those for the wild-type with the same refractory $UO_2$ batch

stoichiometric ratio of nitrate reduced to U(IV) oxidized was effectively identical in the wild-type and the Tbd_0146 and Tbd_0187 mutants, as they all conformed well to a single linear regression model (slope = 21 mol nitrate consumed/mol U(IV) oxidized; $r^2 = 0.97$; $n = 9$). The stoichiometric ratio is similar to that reported previously for the wild-type strain (Beller 2005) and is consistent with the observation (Beller 2005) that U(IV) oxidation, which only accounts for less than 2% of nitrate consumption, is co-metabolic and unlikely to be coupled with energy conservation in *T. denitrificans*.

Three of the four other mutants tested had U(IV) oxidation activity comparable to that of the wild-type (Table 2). The Tbd_1398 mutant only had 76% of the activity of the wild-type, and thus could be somewhat defective, although experimental factors may detract from the comparability of this mutant to all other strains (Table 2, footnote d). The data in Table 2 demonstrate that U(IV) oxidation results cannot be explained by variations in specific denitrification activity in positive controls or by the amount of protein used in a given experiment. For example, the wild-type and Tbd_0187 mutant had protein contents that agreed within 5% but U(IV) oxidation activities that differed by more than a factor of two. Similarly, the Tbd_0187 and Tbd_0723 mutants had identical specific denitrification activities but U(IV) oxidation activities that differed by a factor of more than 2.5. Overall, the correlations between U(IV) oxidation activity and either specific denitrification activity or protein content were very poor ($r^2$ values of 0.01 and 0.07, respectively), which demonstrates that these factors had no substantial influence on the U(IV) oxidation results. The poor correlation between U(IV) oxidation activity and protein content may at first appear counterintuitive, as one would expect greater amounts of relevant enzymes to lead to greater activity (in non-defective strains). However, it seems likely that the rate-determining step of this heterogeneous reaction (i.e., oxidative dissolution of $UO_2$) could be mass transfer of U from the solid to aqueous phase rather than the enzyme-catalyzed redox reaction.

## Homologs of the diheme, *c*-type cytochromes involved in U(IV) oxidation in *T. denitrificans*

Analysis of the translated sequences of Tbd_0187 and Tbd_0146 reveals that both are diheme, *c*-type cytochromes of unknown function (Beller et al. 2006a). The predicted molecular masses and isoelectric points of the unprocessed gene products of Tbd_0187 and Tbd_0146 are 21.4 kDa and 9.2, and 26.7 kDa and 6.3, respectively. BLASTP searches

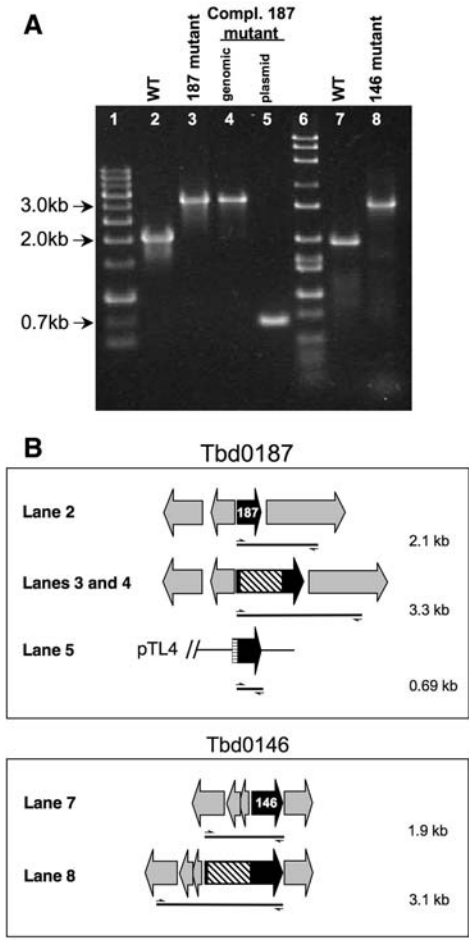


**Fig. 2** PCR-based evaluation of wild-type and mutated versions of the Tbd_0187 and Tbd_0146 genes in wild-type *T. denitrificans*, the two mutants defective in U(IV) oxidation, and the complemented Tbd_0187 mutant. (**a**) Electrophoretic separation of PCR products: *Lane 1*, HyperLadder III, Bioline; *Lane 2*, wild-type DNA, primers Tbd0187ATG-f and 187out-f; *Lane 3*, Tbd_0187 mutant DNA, same primers as Lane 2; *Lane 4*, complemented Tbd_0187 mutant—genomic DNA, same primers as Lane 2; *Lane 5*, complemented Tbd_0187 mutant—plasmid DNA, primers Tbd0187ATG-f and pUC19-f; *Lane 6*, All Purpose Hi-Lo DNA Marker, Bionexus; *Lane 7*, wild-type DNA, primers 0146out-r and 0146-gel-r; and *Lane 8*, Tbd_0146 mutant DNA, same primers as Lane 7 (see Supplemental Table 1 for sequences of PCR primers). (**b**) Map of primer position and amplicon size corresponding to Lanes 2–5 and 7–8 in panel a. Diagonal hatching (Lanes 3, 4, and 8) indicates the kan insertion and horizontal hatching (Lane 5) indicates the kan promoter. Note that the 187out-f primer anneals with genomic DNA downstream of the Tbd_0187 gene, whereas the pUC19-f primer anneals with plasmid DNA from the complemented Tbd_0187 strain (pTL4), rendering these primers specific to the *T. denitrificans* genome and the complementation plasmid pTL4, respectively

(Altschul et al. 1997) of Tbd_0187 and Tbd_0146 versus the genome of *G. metallireducens* [the only other bacterium reported to catalyze nitrate-dependent U(IV) oxidation], did not reveal any hits with E values better than $10^{-5}$ or 20% identity. However, Tbd_0187 is 43% identical at the amino acid level to a well-studied cytochrome $c_4$ from *Pseudomonas stutzeri* (GenBank Q52369); regions of sequence conservation include both CXXCH heme-binding motifs in the translated sequences as well as two methionine residues [Met66 and Met167 in *P. stutzeri*; (Kadziola and Larsen 1997)] that serve as axial ligands for the heme iron atoms. The *P. stutzeri* cytochrome $c_4$ has been characterized biochemically (Hunter et al. 1989; Leitch et al. 1985) and structurally (Kadziola and Larsen 1997) but its exact cellular function has not been described. In *P. stutzeri*, cytochrome $c_4$ is largely membrane-associated and is located on the periplasmic face of the CM; the soluble portion is periplasmic (Hunter et al. 1989). Notably, redox titrations of cytochrome $c_4$ in *P. stutzeri* revealed that the midpoint reduction potentials of the two hemes were +190 and +300 mV (Leitch et al. 1985), which, as noted earlier, is in a range that could accept electrons from $UO_2$. These relatively high reduction potentials are consistent with the His/Met coordination of the two heme iron atoms in cytochrome $c_4$ (involving His18, His123, Met66, and Met167) (Kadziola and Larsen 1997), as His/Met coordination tends to correspond to higher reduction potentials in heme proteins (Reedy et al. 2008). It is likely that His/Met coordination also characterizes the two heme iron atoms in the Tbd_0187 gene product, since the four His and Met residues serving as axial ligands for iron in the *P. stutzeri* cytochrome $c_4$ appear to be conserved in the Tbd_0187 sequence (as mentioned previously). Homologous $c_4$ cytochromes from *Azotobacter vinelandii* and *P. aeruginosa* have midpoint reduction potentials in a similar range as those of *P. stutzeri* (+263 to +322 mV) (Leitch et al. 1985), suggesting that this class of cytochromes (including Tbd_0187) could be amenable to $UO_2$ oxidation and could be positioned close to a terminal oxidase [presumably one associated with denitrification in the case of Tbd_0187, considering the nitrate dependence of U(IV) oxidation].

Tbd_0146 has been annotated as a $c_5$ cytochrome (Beller et al. 2006a), which, like $c_4$ cytochromes,

have been found to be predominantly membrane-associated and periplasm-facing in other bacteria (Hunter et al. 1989). Consistent with membrane association in Tbd_0146, one transmembrane helix has been predicted near the N-terminal region of the protein (residues 16–38 in this 266-residue protein). We are not aware of a close homolog of Tbd_0146 that has been experimentally characterized (e.g., with respect to midpoint reduction potential) or of modeled redox interactions between $c_4$ and $c_5$ cytochromes. Nonetheless, it is plausible that both Tbd_0146 and Tbd_0187 are components of an electron transport chain that ultimately feeds into a terminal reductase, such as nitrite reductase (cytochrome $cd_1$; both hemes C and $D_1$ can have reduction potentials in the range of +290 mV) (Zumft 1997) or nitric oxide reductase (hemes of NorC and NorB can have reduction potentials in the range of +280 and +320 mV, respectively) (Zumft 1997). Regardless of the exact transport chain composition, it is likely that electrons from U(IV) enter at the level of *c*-type cytochromes and it is unlikely that nitrate-dependent U(IV) oxidation results in energy conservation in *T. denitrificans* (Beller 2005), nor indeed is it likely to be of any benefit to the cell. Accordingly, it does not appear that expression of Tbd_0146 and Tbd_0187 depends on exposure to U(IV); whole-genome microarray data (Beller et al. 2006b) (GEO accession number GSE5256) reveal that under thiosulfate-oxidizing, aerobic or denitrifying conditions, expression levels of Tbd_0146 and Tbd_0187 are well within the top 10th or top 5th percentile, respectively, of all genes across the entire genome.

### Residual U(IV) oxidation activity in the defective mutants

Although the mutants of Tbd_0146 and Tbd_0187 were clearly defective in U(IV) oxidation, their residual activity merits discussion. Residual U(IV) oxidation activity in the Tbd_0146 and Tbd_0187 mutants cannot be explained by contamination with the wild-type strain, as we grew the cultures in medium containing 50 μg/ml kanamycin and could not detect wild-type copies of Tbd_0146 or Tbd_0187 in PCR analyses that revealed the prevalence of the mutated genes (Fig. 2). It is also unlikely that the translated product of the disrupted gene was still capable of catalyzing U(IV) oxidation, albeit at a reduced level, because the transposon insertions for these mutants were located upstream of the first heme-binding CXXCH motif; thus, the translated products would not contain any heme groups, which are the sites of electron transfer in cytochromes. Plausible explanations for residual activity in the mutants include (1) two (or more) parallel electron transfer pathways in *T. denitrificans* are capable of accepting electrons from U(IV), (2) upregulation of electron transport proteins in the mutants that partially compensated for the absence of functional proteins encoded by Tbd_0146 or Tbd_0187, or (3) the operative electron transport pathway can still function (albeit less efficiently) when a cytochrome component is missing. For the first two explanations, it is noteworthy that 1–2% of the *T. denitrificans* genome (2827 ORFs) consists of genes encoding *c*-type cytochromes, which is a proportion greater than for almost all bacterial and archaeal species sequenced to date (Beller et al. 2006a). Thus, there is a relatively large pool of *c*-type cytochrome candidates for parallel electron transport pathways or upregulation in defective mutant strains.

## Concluding remarks

The two diheme, membrane-associated *c*-type cytochromes involved in anaerobic U(IV) oxidation in *T. denitrificans* may be periplasmic, based on homology to well-characterized $c_4$ and $c_5$ cytochromes in *P. stutzeri*. If so, the finding that periplasmic rather than OM proteins are involved in the oxidation of $UO_2$ suggests that U(IV) dissolution could occur before U(IV) oxidation, because it is not clear how a sparingly soluble mineral would be available to directly interact with periplasmic proteins. Although the *T. denitrificans* genome contains genes encoding siderophores that might enhance the solubility of U(IV), these genes are poorly expressed under the denitrifying conditions applicable to this study (Beller et al. 2006b) and thus are unlikely to play an important role in nitrate-dependent U(IV) oxidation. Our discovery that the diheme, *c*-type cytochromes Tbd_0187 and Tbd_0146 are involved in anaerobic $UO_2$ oxidation will ultimately facilitate a better understanding of the biochemistry underlying this process and of the biogeochemical dynamics of in situ bioremediation of uranium. For example,

determination of the protein complex partners of Tbd_0187 and Tbd_0146 in electron transport chains will reveal whether an OM protein is involved that directly contacts $UO_2$ and will specify the link to nitrate reduction by identifying the terminal reductase. Furthermore, knowledge of the key genes involved in U(IV) oxidation, as well as U(VI) reduction, will allow us to rigorously monitor these environmentally important and competing processes in situ using PCR-based techniques, as has been done for other bioremediation processes (Beller et al. 2002).

**Acknowledgments** We thank Rachel Lindvall and Edmund Salazar (LLNL) for technical assistance. De novo peptide sequencing was performed by ProtTech, Inc. (Norristown, PA). This work was performed under the auspices of the U.S. Department of Energy by the University of California, Lawrence Livermore National Laboratory under contract No. W-7405-Eng-48.

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