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A New Class of Tungsten-Containing Oxidoreductase in *Caldicellulosiruptor*, a Genus of Plant Biomass-Degrading Thermophilic Bacteria

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***Caldicellulosiruptor bescii* grows optimally at 78°C and is able to decompose high concentrations of lignocellulosic plant biomass without the need for thermochemical pretreatment. *C. bescii* ferments both C_5 and C_6 sugars primarily to hydrogen gas, lactate, acetate, and CO_2 and is of particular interest for metabolic engineering applications given the recent availability of a genetic system. Developing optimal strains for technological use requires a detailed understanding of primary metabolism, particularly when the goal is to divert all available reductant (electrons) toward highly reduced products such as biofuels. During an analysis of the *C. bescii* genome sequence for oxidoreductase-type enzymes, evidence was uncovered to suggest that the primary redox metabolism of *C. bescii* has a completely uncharacterized aspect involving tungsten, a rarely used element in biology. An active tungsten utilization pathway in *C. bescii* was demonstrated by the heterologous production of a tungsten-requiring, aldehyde-oxidizing enzyme (AOR) from the hyperthermophilic archaeon *Pyrococcus furiosus*. Furthermore, *C. bescii* also contains a tungsten-based AOR-type enzyme, here termed XOR, which is phylogenetically unique, representing a completely new member of the AOR tungstoenzyme family. Moreover, in *C. bescii*, XOR represents ca. 2% of the cytoplasmic protein. XOR is proposed to play a key, but as yet undetermined, role in the primary redox metabolism of this cellulolytic microorganism.**

Thermophilic bacteria of the genus *Caldicellulosiruptor* are currently under intense investigation due to their ability to decompose lignocellulosic plant biomass anaerobically at high temperature, thereby potentially mitigating costly thermochemical pretreatment steps (1, 2). One of these species, *Caldicellulosiruptor bescii*, has an optimal growth temperature of 78°C and is the most thermophilic cellulose degrader known to date. It is able to ferment high concentrations of cellulosic feedstock primarily to hydrogen gas, lactate, acetate, and CO_2 (3, 4). Species from this genus can degrade cellulose (and also xylan), using novel multidomain glycosyl hydrolases, representing a new paradigm in cellulose conversion by anaerobic thermophiles (2). Moreover, the recent development of a genetic system for *C. bescii* creates potential for using this and related species for consolidated biomass processing in the production of liquid fuels (5).

Developing metabolic engineering strategies for any microorganism obviously requires an in-depth understanding of their primary metabolism. Evidence that *C. bescii* may have a completely uncharacterized aspect to its primary redox metabolism came from an analysis of its genome sequence for molybdoenzymes (6). These are present in virtually all forms of life, serving diverse roles in primary metabolism of carbon, nitrogen and sulfur (7). As expected, we found that the *C. bescii* genome contains genes necessary for the synthesis of the pyranopterin cofactor that coordinates molybdenum (Mo) in such enzymes (7). Accordingly, the genome also contains a gene (Athe_1215) encoding a member of the dimethyl sulfoxide reductase (DMSOR) family, the most diverse of the three classes of molybdoenzyme (that also includes the xanthine oxidase and sulfite oxidase families [7, 8]). Unexpectedly, however, *C. bescii* contains the *tupABC* operon, which encodes an ABC transporter that is highly specific for the uptake of the analogous metal tungsten (9), instead of the typical *modABC* genes that encode the uptake of molybdenum.

That *C. bescii* might utilize tungsten was very surprising. Although molybdenum-containing enzymes are ubiquitous in biology, microorganisms that require tungsten are extremely limited (10). Indeed, tungsten and molybdenum have such similar chemical and physical properties that almost all microorganisms cannot distinguish between them and often times incorporate tungsten into their molybdoenzymes. This typically renders them nonfunctional (11), although tungsten can be incorporated into some members of the DMSOR family (formate dehydrogenase, formyl methanofuran dehydrogenase, and acetylene hydratase) to yield active enzyme (12). Only a very few microorganisms are known to absolutely require tungsten for growth, and they incor-

Received 17 May 2015 **Accepted** 30 July 2015

Accepted manuscript posted online 14 August 2015

Citation Scott IM, Rubinstein GM, Lipscomb GL, Basen M, Schut GJ, Rhaesa AM, Lancaster WA, Poole FL, II, Kelly RM, Adams MWW. 2015. A new class of tungsten-containing oxidoreductase in *Caldicellulosiruptor*, a genus of plant biomass-degrading thermophilic bacteria. Appl Environ Microbiol 81:7339–7347. doi:10.1128/AEM.01634-15.

Editor: M. J. Pettinari

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.01634-15.

doi:10.1128/AEM.01634-15

TABLE 1 Strains used and constructed in this study

Strain	Parent	Description	Source or reference
Wild type (DSM 6725)		Wild type	39
JWCB018	JWCB005	Δ*pyrFA* Δ*cbeI*	25
MACB1002	JWCB018	Δ*pyrFA* Δ*cbeI* pIMSPFAOR	This study

porate it into the so-called true family of tungstoenzymes represented by aldehyde ferredoxin oxidoreductase (AOR) (12, 13). The AOR family of tungstoenzymes is unrelated phylogenetically to the three families of molybdoenzymes (8, 14), although like the molybdenum in molybdoenzymes, the tungsten in the AOR family is coordinated by a pyranopterin cofactor (14, 15).

The best-characterized microorganisms that contain the AOR family of tungstoenzymes are members of the hyperthermophilic archaea, represented by *Pyrococcus furiosus*, which grows optimally at 100°C. Such organisms have high selectivity for the two metals. For example, *P. furiosus* does not incorporate significant amounts of molybdenum into its AOR, even when the organism is grown in a 40-fold excess of molybdenum over tungsten (16). *P. furious* grows by fermenting sugars (but not cellulose) and peptides and contains five members of the AOR family (abbreviated AOR [17], GAPOR [18], FOR [19], WOR4 [20], and WOR5 [21]), all of which oxidize aldehydes of various types. The prototypical AOR has a broad substrate specificity and is thought to be involved in peptide catabolism wherein it oxidizes amino acid-derived aldehydes (10).

It was therefore surprising to find that the *C. bescii* genome not only encodes a tungstate transporter but also contains a gene (Athe_0821) annotated as a member of the AOR family. This suggests that *C. bescii* possesses a previously unknown ability to utilize tungsten. Here, we show that this is indeed the case by demonstrating heterologous production of active, tungsten-containing *P. furiosus* AOR in *C. bescii*. Moreover, the "AOR" of *C. bescii* is phylogenetically unique and represents a sixth distinct member of the AOR family. This new type of tungstoenzyme is proposed to play a key role in the primary redox metabolism of this cellulolytic microorganism.

MATERIALS AND METHODS

Strains and growth conditions. *C. bescii* strains used or constructed in the present study are listed in Table 1. Low-osmolarity defined (LOD) medium (22) was prepared from filter sterilized stock solutions. The 50× base salts solution contained 16.5 g of $MgCl_2$, 16.5 g of KCl, 12.5 g of NH_4Cl, 7 g of $CaCl_2 \cdot 2H_2O$, and 0.68 g of KH_2PO_4 per liter. Trace element solution SL-10 is prepared as described previously (23), and the 200× vitamin solution contained the following vitamins (in milligrams) per liter: biotin, 4; folic acid, 4; pyridoxine-HCl, 20; riboflavin, 10; thiamine-HCl, 10; nicotinic acid 10; pantothenic acid, 10; vitamin B_{12}, 0.2; *p*-aminobenzoic acid, 10; and lipoic acid, 10. Unless otherwise indicated, *C. bescii* was routinely cultured under strict anaerobic conditions at 75°C with shaking at 200 rpm in LOD medium (22), with the exception that maltose was replaced with cellobiose and sodium molybdate and sodium tungstate were added at final concentrations of 1 μM. *Pyrococcus furiosus* strain COM1 (24) was cultured under strict anaerobic conditions at 90°C in static bottles in artificial seawater medium containing per liter: 5 g of maltose, 1× base salts (23), 1× trace minerals (23), 10 μM sodium tungstate, 0.25 μg of resazurin, 2 g of yeast extract, 0.5 g of cysteine, 0.5 g sodium sulfide, 1 g of sodium bicarbonate, 1 mM potassium phosphate buffer (pH 6.8), and 20 μM uracil.

RNA isolation and quantitative reverse transcription-PCR (RT-PCR). *C. bescii* was grown in 100-ml sealed serum bottles with 50 ml of LOD medium containing cellobiose (5 g $liter^{-1}$) at 75°C until mid-exponential phase (optical density at 680 nm of 0.06 to 0.08). Cultures were cooled to 4°C, cells were harvested by centrifugation, and cell pellets were stored at −80°C. For total RNA isolation, frozen cell pellets were suspended in 300 μl of lysis buffer (4 M guanidine thiocyanate, 0.83% *N*-lauryl sarcosine [pH 5]), followed by the addition of 300 μl of acid-equilibrated phenol-chloroform (5:1; pH 4.3 to 4.7; Sigma). After vortexing the tubes to form an even suspension, the suspended cells were subjected to three 10-s intervals of sonication (amplitude 40; Qsonica Q55), interspaced by at least 30 s. The resulting cell lysate was mixed with 600 μl of 100% ethanol, and total RNA was isolated using a Direct-Zol RNA MiniPrep kit (Zymo Research), according to the manufacturer's protocol, with the exception that genomic DNA was digested in solution as opposed to on the column using Turbo DNase (Ambion). RNA was quantified with a Nano-Drop 2000c spectrometer (Thermo Scientific). Synthesis of cDNA was performed with 1 μg of purified RNA using the Affinity Script QPCR cDNA synthesis kit (Agilent). A Brilliant II SYBR green QPCR master mix (Agilent) was used for quantitative reverse transcription-PCR (RT-PCR) experiments with primers designed to amplify a ~200-base product within the target genes: Athe_0821 (*xor*) and PF0346 (*aor*). For comparison to the Athe_1406 gene (the GAPDH gene) which is expressed at high levels during growth on Avicel, cellobiose, glucose, xylose, and xylan using RNA-seq (data not shown). The primers used in the present study are presented in Table 2.

Phylogenetic analysis. BLAST searches of the amino acid sequences of XOR and the unknown dehydrogenase (UDH) were performed against the NCBI database using the default settings. The top 2,000 hits for XOR and the top 10,000 hits for UDH were used to construct phylogenetic trees for each on the basis of neighborhood joining and Jukes-Cantor methods, with 100 bootstrap replicates done for each tree using CLC Main Workbench 6 (CLC Bio).

Plasmid construction. The *C. bescii* replicating shuttle vector pDCW89 (25) was modified via Gibson Assembly (New England BioLabs) to include a His tag and a multiple cloning site from the commercial vector pET24a (Novagen), generating pIMS89. The *P. furiosus aor* gene (PF0346) and the *C. bescii* S-layer protein promoter region (200 bp starting immediately upstream of the start of Athe_2303) were amplified from genomic DNA, spliced together using overlap PCR and cloned into pIMS89 to create pIMSAOR (see Fig. S1 in the supplemental material).

Strain construction. *C. bescii* strain JWCB018 cells were rendered competent as previously described (26) and transformed using 0.5 μg of purified plasmid pIMSAOR via electroporation by a single electric pulse (2.0 kV, 25 μF, and 200 Ω) in a 1-mm cuvette using a Gene Pulser (Bio-Rad). Transformants were allowed to recover at 75°C in 20 ml of LOC medium (22) for 1 to 2 h, after which cells from 1 ml of the recovery culture were harvested and transferred to LOD medium lacking uracil. These selective outgrowth cultures were incubated 18 to 72 h, and those with appreciable growth were colony purified on solid LOD medium lacking uracil. The strain was verified to contain the pIMSAOR plasmid by PCR screening, as well as backtransforming isolated plasmid into *E. coli*. The purified strain was designated MACB1002 (Table 1).

Preparation and fractionation of cell extracts for metal analysis. *C. bescii* strains JWCB018 and MACB1002 were each cultured in 4 liters of LOD medium and harvested in the late-exponential phase by centrifugation at 6,000 × *g* for 10 min (Beckman Avanti J-30I JLA 10.500 rotor) to yield 3.8 and 3.1 g of cells (wet weight), respectively. Cell pellets were flash frozen in liquid nitrogen and stored at −80°C. Strict anaerobic conditions were maintained for all successive steps. Cell pellets were thawed and suspended in 25 mM Tris buffer (pH 8.0) containing 1 mg ml^{-1} lysozyme (Sigma-Aldrich) in a ratio of 3 ml per g of cells. The suspended cells were incubated at room temperature for 15 min followed by three 10-s intervals

TABLE 2 Primers used in this study

Primer	Target locus[a]	Sequence (5′–3′)
pEThtclo F	pET24a	CCAATGATCGAAGTTAGGCTGGTATCCGGATATAGTTCCTCCTTTCAGC
pEThtclo R	pET24a	GCTCACGGTGGTACCGCGGCCGCATGCTCCCGGGATCCCACCACCACCACCACTGAG
pims89 F	pDCW89	CTCAGTGGTGGTGGTGGTGGGATCCCGGGAGCATGCGGCCGCGGTACCACCGTGAGC
pims89 R	pDCW89	GCTGAAAGGAGGAACTATATCCGGATACCAGCCTAACTTCGATCATTGG
PF-AORsphI R2	*P. furiosus aor*	AAAAAGCATGCATAGAACTCAGCGATTCCAAGC
PF-AOR F	*P. furiosus aor*	GGAGGTTTGGTGAGTAGTTATGTATGGCAACTGGGG
PFaorSporR	*slp* promoter region	CCCCAGTTGCCATACATAACTACTCACCAAACCTCC
KpnISpro F	*slp* promoter region	AAAGGTACCACAGGATTTAAAAGAGGCTATGCAG
GL313	*P. furiosus aor*	CATCAAAGACGAGCACATTGAG
GL314	*P. furiosus aor*	GCGAACTTGACCAAATTCTCAC
Athe_0820qF	Fd gene	ACAAGAGGAGGACTTCAGAGT
Athe_0820qR	Fd gene	TTCTCACACATGCTCCACAG
Athe_0821qF	*xor*	CTTGGGATTGGTACGGTCTTT
Athe_0821qR	*xor*	GTGTTCCAAGCTCGTGATACT
Athe_0823qF	*moeB*	TTGTTGGAGTGGGTGGAATAG
Athe_0823qR	*moeB*	AGAAACGGCAGGGTTTATCTC
Athe_0824qF	*tupA*	GCTTATAAATGGTTATACTGCG
Athe_0824qR	*tupA*	GCTGAACCTTTGCTAACTTCC
Athe_0825qF	*tupB*	CCTATAGTGATGCTTTATACCCTGTC
Athe_0825qR	*tupB*	TCCTTCTATGTTACCTCCAACAATC
Athe_0826qF	*tupC*	CAGGGCGGATACACAATCTG
Athe_0826qR	*tupC*	CCCAGCTGAGAGCTGATTTATG
Athe_0827qF	*moeA1*	GTCATCCTGTGTCTTGCCTT
Athe_0827qR	*moeA1*	CTGATTTTCCATAAAGAGGCTCAG
Athe_0828qF	*moeA2*	GTTTACAGGGCGAGGATATAAAAG
Athe_0828qR	*moeA2*	GAACTCAACAAGCTGTCCAAC
Athe_0829qF2	*moaA*	CGCTCTGTGAACCTTGATGAG
Athe_0829qR2	*moaA*	GAAACTTCCACTGTCCCTATTTC
Athe_0830qF	*moaC*	GTATGACATGTGCAAAGCGG
Athe_0830qR	*moaC*	CATCTCCTTCAATCCCATAGTC
Athe_0831qF	*moaB*	CGAACCTGGAAGATTTATTATCAAACTG
Athe_0831qR	*moaB*	CTGACAACTGGTGGAACAGG
Athe_1082qF	*mbh*	GGAACGGTTGCTTTGAATGAG
Athe_1082qR	*mbh*	CTATTCTTCCCAGCCATGTATCA
Athe_1215qF	*udh*	ACCTTGCCTATGGAGCAATAAA
Athe_1215qR	*udh*	TCTTTGCGGACCATATCCAATAA
Athe_1216qF	*mobA*	GGGCATATAGAGTTTGAAGGG
Athe_1216qR	*mobA*	GCCATTATAATAAGGAACAACTGC
Athe_1299qF	*hydA*	CGTGGCCTTGAAGGAATAAGA
Athe_1299qR	*hydA*	CACCGGGACATGCCATAAT
Athe_1406qF	GAPDH gene	GCTGCAGAAGGCGAATTAAAG
Athe_1406qR	GAPDH gene	GGTCTGCAACTCTGTTGGAATA
Athe_2303qF	*slp* promoter region	TGCGACTGAGCGAAGATAAG
Athe_2303qR	*slp* promoter region	CTTTACTATAGCCGGAGCTGTT

[a] Primers are targeted to *C. bescii* genome loci unless otherwise noted.

of sonication (amplitude 40; Qsonica Q55) interspaced by at least 30 s. Cell lysates were clarified by ultracentrifugation at 100,000 × *g* for 1 h (Beckman L90K ultracentrifuge 70.1Ti rotor). Clarified cell lysates from each cell batch were directly loaded onto a 1 ml Hi-trap QHP column (GE Healthcare) equilibrated anaerobically with 25 mM Tris buffer (pH 8.0), using an ÄKTA purifier system (GE Healthcare). Protein was eluted using a linear gradient of 0 to 1 M NaCl in 25 mM Tris buffer (pH 8.0), and fractions were collected anaerobically. Metal concentrations were measured in the cytoplasmic extract and in column fractions using quadrupole-based inductively coupled plasma mass spectroscopy (ICP-MS) as described previously (27). Proteins were identified in the chromatography fractions by liquid chromatography-tandem mass spectrometry (Proteomics and Mass Spectrometry Facility, University of Georgia).

Purification of heterologously expressed AOR from *C. bescii*. All purification steps were performed under strictly anaerobic conditions. Frozen cell pellets (3.5 g) of *C. bescii* strain MACB1002 were lysed and a cell-free supernatant (13 ml containing 31.0 mg of protein ml^{-1}) was obtained as described above. This was applied to a 1-ml HisTrap excel column (GE Healthcare) equilibrated in buffer A (50 mM phosphate buffer [pH 7.2], 300 mM NaCl). The column was washed with five volumes of buffer A, and AOR was eluted with a gradient of 0 to 500 mM imidazole in buffer B (buffer A containing 500 mM imidazole) over 20 column volumes. Fractions were collected in 2-ml volumes in sealed serum bottles made anaerobic by degassing with argon. Fractions containing AOR activity were analyzed for purity using denaturing SDS-PAGE gradient electrophoresis (4 to 12% Bis-Tris gels; Novex) and stained with Imperial protein stain (Novex). Purified protein was analyzed for metal content via ICP-MS as described previously (27).

Enzyme assays. The aldehyde oxidizing activity of cell extracts of *C. bescii* strains JWCB018 and MACB1002 and *P. furiosus* COM1 were determined by measuring the reduction of benzyl viologen (1 mM) in 50 mM EPPS buffer (pH 8.0) at 75°C in rubber-stoppered cuvettes under

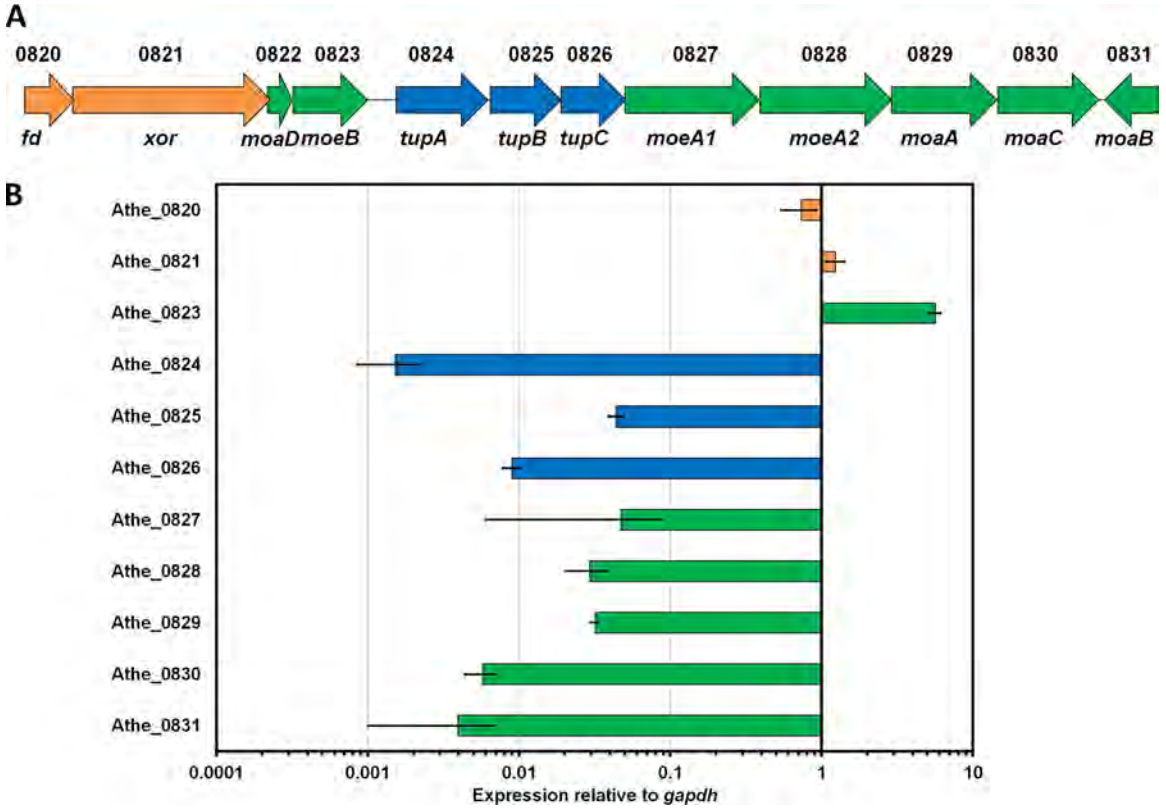

FIG 1 (A) *C. bescii* gene cluster (Athe_0820 to Athe_0831) encoding the proteins necessary for pyranopterin biosynthesis (green) and tungstate transport (blue), as well as a ferredoxin (orange) and XOR (orange). (B) Expression levels of the genes encoding pyranopterin biosynthesis (*moeB, moeA1*, *moeA2*, and *moaABC*), tungstate transport (*tupABC*), ferredoxin (Fd gene) and XOR (*xor*) relative to that of the gene encoding the glycolytic enzyme GAPDH, as determined by quantitative RT-PCR. Bars are color-coded according to genes in part A. Error bars represent the standard deviations (SD; $n = 3$ technical replicates).

anaerobic conditions using various aldehydes (1 mM) as the substrates. To remove trace amounts of O_2, sodium dithionite was added to the assay mixture to give an A_{600} of ~0.2. The extract was added and, after a 1-min incubation period, the reaction was initiated by addition of the aldehyde. An extinction coefficient of 7.4 $mM^{-1}cm^{-1}$ was used for reduced benzyl viologen (28). Specific enzyme activities are expressed as units per mg of protein, where one unit represents 1 μmol of aldehyde oxidized per min.

RESULTS

Analysis of the tungsten pyranopterin biosynthetic gene cluster in *C. bescii*. An analysis of the *C. bescii* genome revealed that it contains all but one of the genes necessary for the synthesis of the pyranopterin cofactor from GTP (29). These are arranged in a gene cluster (Athe_0822 to Athe_08031) that includes *moaABCD*, *moeA1*, *moeA2*, and *moeB* (Fig. 1A). Absent from the genome of *C. bescii* is a gene encoding MoaE. This is very unusual, but not without precedent in microorganisms that utilize tungsten (30). Another unusual feature is the presence of two genes encoding *moeA* homologs in the *C. bescii* genome. It has been hypothesized that MoeA functions in metal selectivity between tungsten and molybdenum, but a mechanism for this selectivity has yet to be elucidated (31). As shown in Fig. 1A, instead of the expected *modABC* genes, which encode a molybdate transporter, the *tupABC* genes are found, and these encode a transporter specific for tungstate. The relative expression levels of the pyranopterin biosynthesis and tungstate-related genes in wild-type *C. bescii* grown on cellobiose in the presence of 1 μM tungstate and 1 μM molybdate are shown in Fig. 1B. All of the genes are expressed at least an order of magnitude lower than that of the gene encoding the glycolytic enzyme, glyeraldehyde-3-phosphate dehydrogenase (GAPDH), with the exception of *moeB*, which is encoded within a separate upstream gene cluster. This gene is at least 20-fold higher in expression than the GAPDH gene, and perhaps higher expression is needed because of the role of MoeB in recycling MoaD (8, 32).

Analyses of *C. bescii* tungsten utilization by heterologous expression of AOR. To determine whether the pyranopterin and tungstate-related genes in *C. bescii* were fully functional and could support the uptake of tungstate and its incorporation into a known tungstoenzyme, the organism was engineered to heterologously express an affinity-tagged version of AOR from *P. furiosus*, one of the most well-characterized tungstoenzymes (14). The gene encoding *P. furiosus* AOR (PF0346) was modified to include an N-terminal polyhistidine tag, and its expression was placed under the control of the promoter of the gene (*slp*) encoding the S-layer protein of *C. bescii*. Under standard growth conditions, the *slp* gene is expressed at a level that is about 10-fold higher than that of the GAPDH gene in wild-type *C. bescii* (Fig. 2A). The *aor* expression construct was inserted into a shuttle vector (see Fig. S1 in the supplemental material), and this was transformed into the genetic background strain JWCB018 (33) to create strain MACB1002 (Table 1). Analysis of the AOR gene expression level in strain MACB1002 revealed that it was higher than *slp* (Fig. 2A). In spite of the extremely high expression level of *aor*, strain MACB1002

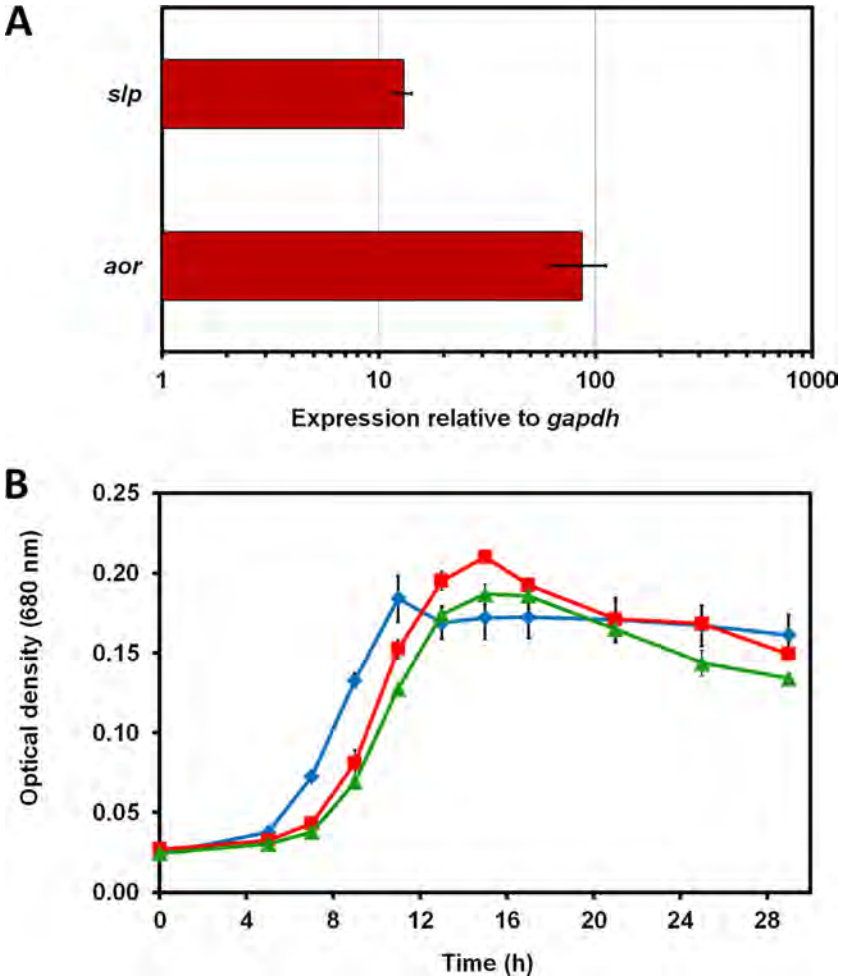

FIG 2 High-level expression of AOR in *C. bescii* does not affect growth. (A) Quantitative RT-PCR expression levels of *C. bescii slp* and *P. furiosus aor* in *C. bescii* strain MACB1002 relative to the gene encoding the glycolytic enzyme GAPDH. (B) Growth curves of wild-type (green triangles), genetic background strain JWCB018 (red squares), and *P. furiosus* AOR-expressing strain MACB1002 (blue diamonds) grown on LOD medium supplemented with 1 μM tungsten and 1 μM molybdenum. The error bars represent the SD ($n = 3$).

exhibited no obvious growth phenotype when cultured on a cellobiose-containing medium with 1 μM W and 1 μM Mo (Fig. 2B).

AOR is highly expressed in *P. furiosus*, and in *in vitro* assays exhibits high activity using acetaldehyde (1.0 mM) as the substrate with the dye benzyl viologen as the electron acceptor (17). The specific activity in a cell extract of *P. furiosus* was 4.0 ± 0.1 U/mg at 75°C. The cell extract of the parent *C. bescii* strain contained no detectable AOR activity (<0.01 U/mg), using acetaldehyde as the substrate. However, the cell extract of the MACB1002 strain, harvested at the end of exponential growth, contained 3.5 ± 1 U/mg, showing that the *P. furiosus* enzyme was produced in *C. bescii* at a level comparable to that in its native organism.

P. furiosus AOR was purified from a cell extract of *C. bescii* strain MACB1002 by a single affinity chromatography step, yielding an enzyme that was close to homogeneity by SDS-PAGE analysis (Fig. 3). Although AOR is a homodimeric enzyme, it is known to migrate as two bands corresponding to the denatured monomeric and the undenatured dimeric forms of the enzyme (17). Purification resulted in relatively high recovery of AOR activity (45%; Table 3), and the specific activity of purified AOR with acetaldehyde (49 U/mg) was comparable to that measured with

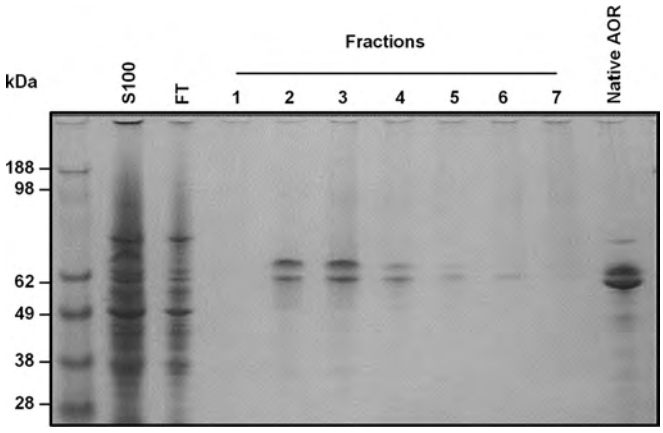

FIG 3 SDS-PAGE analysis of *P. furiosus* AOR purified from *C. bescii* strain MACB1002 by a single affinity chromatography step. Gel annotations are as follows: S100, cytoplasmic fraction; FT, flowthrough; fractions, nickel-NTA elution fractions 1 to 7; native AOR, AOR purified from *P. furiosus*. The enzyme exhibits two bands on an SDS gel corresponding to the denatured monomeric and the undenatured dimeric forms, as previously observed (17).

TABLE 3 Purification of *P. furiosus* AOR from *C. bescii* strain MACB1002

Sample[a]	Protein (mg)	Activity (U)	Sp act (U/mg)	Recovery (%)
JWCB018 CE	186	<0.1	<0.1	
P. furiosus CE			4.0	
MACB1002 CE	312	167	3.5	100
AC flowthrough	259	1.5	0.1	2.4
AC elution	20	194	56	52

[a] Abbreviations: CE, cytoplasmic extract; AC, affinity chromatography.

native *P. furiosus* AOR (17). This enzyme has been shown to be dimeric with each subunit containing one W and four Fe per monomer and an additional iron per monomer that is shared between the two subunits (14). ICP-MS analysis of *P. furiosus* AOR from *C. bescii* yielded an iron to tungsten ratio (Fe/W) of 4.25 ± 0.25, which is close to the value of 4.5 for the pure enzyme. Purified AOR contained only trace amounts of molybdenum with a W/Mo ratio of 62:1. These results, therefore, demonstrate that when *C. bescii* is grown in the presence of 1 μM W and 1 μM Mo, it is highly selective for tungsten, similar to *P. furiosus*. Moreover, the level of expression of the pyranopterin and tungstate-related genes in *C. bescii* can generate a high cellular concentration of recombinant *P. furiosus* AOR that is very active, contains tungsten rather than molybdenum in its active site, and has the characteristics of natively purified AOR.

***C. bescii* contains an AOR homolog.** As shown in Fig. 1A, adjacent to the genes encoding pyranopterin biosynthesis and tungstate transport in *C. bescii* are two genes that are annotated as aldehyde ferredoxin oxidoreductase (Athe_0821) and ferredoxin (Athe_0820). Athe_0821 encodes a protein (586 residues) that shows 31% sequence similarity to *P. furiosus* AOR (605 residues), and Athe_0820 is predicted to encode a polyferredoxin with the potential to contain four [4Fe-4S] clusters according to its cysteine motifs. This 12-gene cluster (Athe_0820 to Athe_0831) is conserved in the genomes of the eight *Caldicellulosiruptor* species that have been sequenced to date, and there is high identity among the AOR homologs (94 to 99%: see Table S1 in the supplemental material), suggesting that the AOR homolog has an important role in this group of microorganisms.

In *C. bescii*, the genes encoding the AOR homolog (Athe_0821) and its associated ferredoxin (Athe_0820) are among the most highly transcribed genes during growth on glucose, cellobiose, cellulose, and switchgrass, and they do not appear to be significantly regulated under any of these growth conditions, according to DNA microarray data (3). As shown in Fig. 1B, quantitative PCR analysis shows that during growth on cellobiose the genes encoding the AOR homolog and the ferredoxin are expressed at levels similar to that of the gene encoding the glycolytic enzyme GAPDH, and these are an order of magnitude higher than those genes encoding pyranopterin biosynthesis and tungstate transport (Fig. 1B). As noted above, the cytoplasmic fraction of *C. bescii* cells did not contain significant acetaldehyde-oxidizing activity, which is a characteristic of *P. furiosus* AOR. The other four members of the AOR family of tungstoenzymes, GAPOR, FOR, WOR4, and WOR5, oxidize a range of other aldehydes, including formaldehyde, propionaldehyde, crotonaldehyde, glutaraldehyde, isovaleraldehyde, benzaldehyde, and glyceraldehyde-3-phosphate (17–21). However, the cell extract of the parent *C. bescii* strain did not oxidize any of these aldehydes at detectable rates (<0.01 U/mg at 75°C). Given its high expression level in *C. bescii*, we conclude that the AOR homolog encoded in its genome does not directly correspond to any of the five known members of the AOR family. Henceforth, the *C. bescii* AOR homolog will be referred to as XOR to indicate that its physiological substrate is not known.

In order to provide additional insights into the role of XOR and tungsten in the metabolism of *C. bescii*, a cell extract was fractionated by anion-exchange chromatography and the fractions were analyzed for tungsten and molybdenum by ICP-MS. As shown in Fig. 4, a large tungsten peak was observed, which overlaid a minor peak of molybdenum representing ca. 5% of the tungsten. Analysis of the peak tungsten-containing fractions by MS/MS revealed that XOR was a major protein, as indicated by almost complete coverage of the protein by the peptides that were detected (see Fig. S2 in the supplemental material). All members of the AOR family

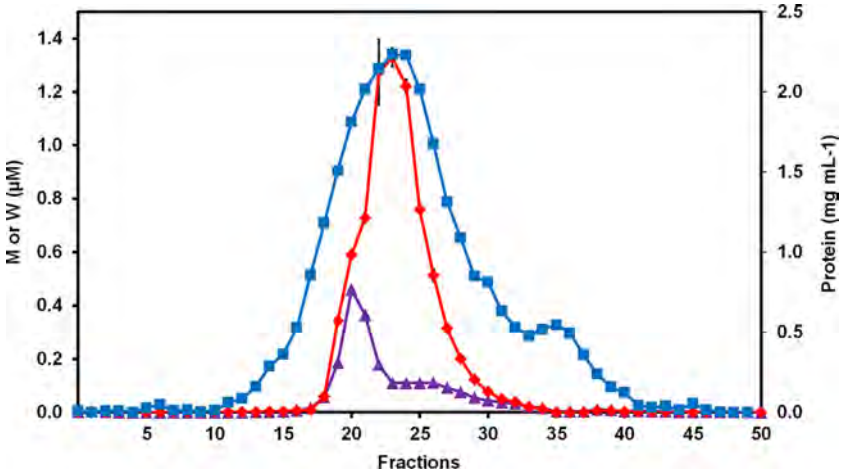

FIG 4 Elution profile of tungsten (red diamonds), molybdenum (purple triangles), and protein (blue squares) after fractionation of a cytoplasmic extract of *C. bescii* strain JWCB018 by anion-exchange chromatography. The cells were grown in LOD medium supplemented with 1 μM tungstate and 1 μM molybdate. Error bars for tungsten and molybdenum curves represent the SD ($n = 3$ technical replicates).

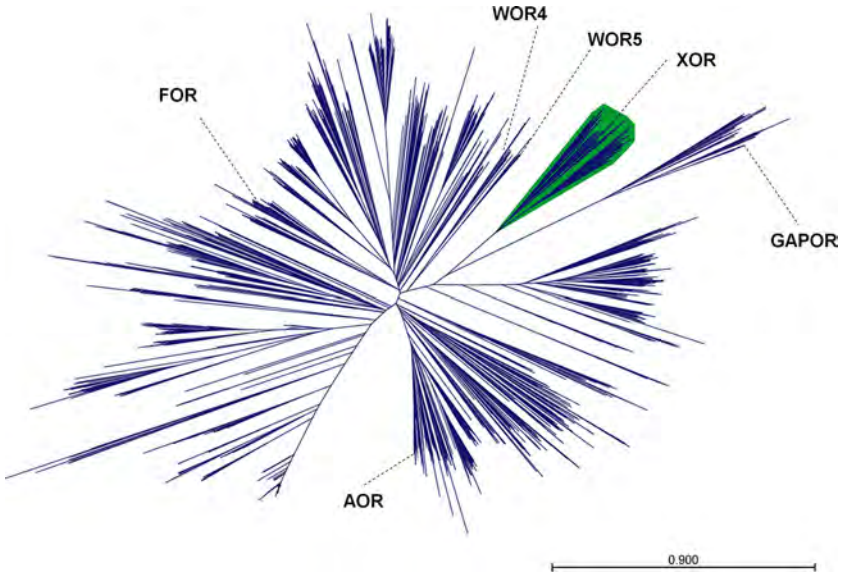

FIG 5 Phylogenetic tree of 2,000 AOR family homologs. The characterized enzymes from *P. furiosus* are indicated: formaldehyde ferredoxin oxidoreductase (FOR) (19), glyceraldehyde ferredoxin oxidoreductase (GAPOR) (18), aldehyde ferredoxin oxidoreductase (AOR) (17), and tungsten oxidoreductases of unknown function WOR4 and WOR5. The clade containing XOR is highlighted green to include homologs predicted across other species using the STRING database (20, 31). The protein sequences used to construct the tree were selected from protein BLAST hits of Athe_0821 against the NCBI database. The scale bar indicates the Jukes-Cantor distance of sequences on the tree.

consist of a single subunit of approximately the same size (~65 kDa) that contains a single tungsten atom bound by the pyranopterin cofactor (17–21). Given that XOR is of similar size and shows sequence similarity to the other AOR members, it is reasonable to assume it also contains a single tungsten atom. Hence, if XOR is the only tungsten-containing protein in the tungsten elution profile (Fig. 4), it represents ca. 1.9% of the cytoplasmic protein applied to the anion-exchange column.

Phylogenetic analysis of the top 2000 BLAST hits for *C. bescii* XOR reveals that this enzyme is very distinct from the other five characterized members of the AOR family of enzymes. Moreover, as shown in Fig. 5, the XOR branch forms a very distinct and separate clade, a finding consistent with a function that is distinct from that of the other AOR family members. The characterized enzymes also fall in distinct clades (with WOR4 and WOR5 within a single clade). This analysis also reveals that there are at least three other large clades within the AOR family of enzymes about which little is known since there are no characterized representatives (Fig. 5). Further analysis of the gene synteny surrounding *xor* within the XOR clade shows that, although the gene cluster encoding proteins for pyranopterin biosynthesis and tungstate transport are not conserved, the gene encoding the polyferredoxin is always found adjacent to the XOR homolog (see Fig. S3 in the supplemental material). Hence, the polyferredoxin might be the electron carrier for XOR, or it could potentially be a subunit of a heterodimeric XOR enzyme. However, peptides attributable to the polyferredoxin (Athe_0820) were not detected in the fractions containing XOR (Athe_0821) after anion-exchange chromatography of a cell extract (Fig. 4).

To determine whether XOR was essential for growth of *C. bescii* on cellulose, an attempt was made to delete *xor* from the genome. Transformants for chromosomal integration of the *xor* knockout plasmid containing the *pyrF* marker were selected using uracil prototrophy, and counterselection for plasmid loss and gene deletion was performed using resistance to 5-fluoroorotic acid, as illustrated in Fig. S4 in the supplemental material. This same strategy was used to successfully knock out the gene encoding lactate dehydrogenase in the same genetic background strain (34). However, under these conditions, XOR deletion was not successful. Although recombination of the plasmid into the *xor* flanking region was verified, counterselection for plasmid loss resulted in reversion to the wild-type allele, and not *xor* deletion, in more than 200 screened isolates. Altogether, these results indicate that XOR plays a key role in *C. bescii* metabolism.

***C. bescii* contains a DMSOR homolog.** In addition to the gene cluster encoding putative tungsten-containing XOR, polyferredoxin, pyranopterin synthesis, and tungstate transport, the *C. bescii* genome also contains a gene encoding a member of the dimethyl sulfoxide reductase (DMSOR) family of molybdoenzymes (Athe_1215), as well as an adjacent gene encoding the pyranopterin guanine dinucleotide synthesis gene *mobA* (Athe_1216). These two genes are located remotely from the XOR gene cluster, and they are also present as adjacent genes in all eight of the other available *Caldicellulosiruptor* genome sequences. The guanine dinucleotide form of pyranopterin is required for DMSOR family enzymes. Expression levels of Athe_1215 measured by qPCR show that it is expressed at ca. 10% of the level of the GAPDH gene (see Fig. S5 in the supplemental material). Microarray data suggest that Athe_1215 is not significantly regulated on any of the tested substrates (3). This DMSOR homolog was not detected via MS/MS analysis in either the Mo- or W-containing peaks of fractionated cell extract (Fig. 4), although this may be due to the lower expression level relative to the GAPDH gene compared with that of *xor*. Phylogenetic analysis reveals that the protein coded by this gene belongs to a subclass of the DMSOR family that is related to, but distinct from, well-characterized molybdoenzymes such as DMSOR, formate dehydrogenase, assimilatory and periplasmic nitrate reductases, trimethyl N-oxide reductase, and biotin sulfox-

ide reductase (see Fig. S6 in the supplemental material). This protein of unknown function does not resemble any of the characterized clades of DMSOR family enzymes and henceforth will be referred to as UDH for unknown dehydrogenase.

DISCUSSION

We show herein that *C. bescii* contains a gene cluster that encodes pyranopterin synthesis and tungstate transport. In addition, we demonstrate that *C. bescii* can heterologously express the *P. furiosus* tungsten-containing enzyme AOR at a high cellular concentration and with no apparent growth phenotype. The ability of *C. bescii* to express this AOR demonstrates that it has a very active tungsten utilization pathway, an important factor to consider for future engineering strategies in this organism. For example, the recently discovered alcohol production pathway from organic acids, involving AOR and an aldehyde dehydrogenase, AdhA (35), might be applicable in this organism and other *Caldicellulosiruptor* species.

Adjacent to the pyranonpterin biosynthetic gene cluster in the *C. bescii* genome is the gene encoding an AOR family enzyme for which function has yet to be determined and is therefore termed XOR. We show that *xor* is highly expressed in *C. bescii*, and it likely represents the major, if not the only, tungsten-containing enzyme within the cell. Phylogenetic analyses of the AOR family (Fig. 5) revealed that XOR is part of a unique clade distinct from the other characterized members. Surprisingly, this also revealed that there are at least three other major classes of the AOR family that remain to be functionally characterized, in addition to XOR. Of the five characterized members of the AOR family, only GAPOR has a well-defined physiological role. It replaces the conventional glycolytic enzyme GAPDH in some hyperthermophilic archaea and oxidizes glyeraldehyde-3-phosphate using ferredoxin rather than NAD as the electron acceptor (18). Of the others family members, AOR and the formaldehyde-oxidizing FOR are thought to function in peptide catabolism (19, 36). The functions of WOR4 and WOR5 remain unknown (20, 21).

Only a very few other microorganisms, including some acetogens and some ethanol- and phenylalanine-oxidizing anaerobes (12, 37, 38), also contain members of the AOR family, but these are all the prototypical type of AOR represented by *P. furiosus* AOR. This enzyme has broad substrate specificity and catalyzes the reversible oxidation of both aliphatic and aromatic aldehydes derived from amino acid metabolism. *C. bescii* is distinctive, since its AOR family member, XOR, does not utilize (at least in cytoplasmic extracts) the aldehydes oxidized by *P. furiosus* AOR, nor indeed by any other characterized member of the AOR family. Unfortunately, insight into the function of *C. bescii* XOR is not evident from microorganisms that contain XOR. As indicated in Fig. S3 in the supplemental material, they are quite diverse and include sulfate-reducing bacteria and methanogenic and sulfate-reducing archaea. The only common feature is that they are all anaerobic microorganisms. Hence, the designation of the *C. bescii* AOR homolog as XOR seems appropriate.

Although its role within the cell is as yet unknown, the conservation of XOR and its associated polyferredoxin across the genus *Caldicellulosiruptor*, together with its high expression level in *C. bescii* (~2% of the cytoplasmic protein), suggest that this novel tungstoenzyme serves an important role in the primary metabolism of these cellulolytic species. Determining its function will likely have an important impact on future metabolic engineering studies of *C. bescii*, and studies to elucidate the substrate(s) utilized by XOR are under way.

ACKNOWLEDGMENTS

We thank Jeffrey Zurawski, Jonathan Conway, and Laura Lee for many helpful discussions and Daehwan Chung and Janet Westpheling for providing strains, plasmids, and protocols for genetic manipulation of *C. bescii*. We also acknowledge the University of Georgia Proteomics and Mass Spectrometry Facility for performing mass spectrometry analysis and computational support.

This research was supported by the U.S. Department of Energy's BioEnergy Science Center (BESC) through the Office of Biological and Environmental Research.

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