Rex in *Caldicellulosiruptor bescii*: Novel regulon members and its effect on the production of ethanol and overflow metabolites

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Abstract

Rex is a global redox-sensing transcription factor that senses and responds to the intracellular [NADH]/[NAD⁺] ratio to regulate genes for central metabolism, and a variety of metabolic processes in Gram-positive bacteria. We decipher and validate four new members of the Rex regulon in *Caldicellulosiruptor bescii*: a gene encoding a class V aminotransferase, the HydG FeFe Hydrogenase maturation protein, an oxidoreductase, and a gene encoding a hypothetical protein. Structural genes for the NiFe and FeFe hydrogenases, pyruvate:ferredoxin oxidoreductase, as well as the rex gene itself are also members of this regulon, as has been predicted previously in different organisms. A *C. bescii rex* deletion strain constructed in an ethanol-producing strain made 54% more ethanol (0.16 mmol/L) than its genetic parent after 36 hr of fermentation, though only under nitrogen limited conditions. Metabolomic interrogation shows this rex-deficient ethanol-producing strain synthesizes other reduced overflow metabolism products likely in response to more reduced intracellular redox conditions and the accumulation of pyruvate. These results suggest ethanol production is strongly dependent on the native intracellular redox state in *C. bescii*, and highlight the combined promise of using this gene and manipulation of culture conditions to yield strains capable of producing ethanol at higher yields and final titer.

KEYWORDS

*Caldicellulosiruptor bescii*, consolidated bioprocessing, ethanol, Rex
1 INTRODUCTION

Liquid transportation fuel demand is projected to increase through 2075 (Fulton, Lynd, Körner, Greene, & Tonachel, 2015) and an inexpensive, reliable way to produce bioethanol from lignocellulosic feedstocks will be necessary to meet increased demand. Consolidated bioprocessing (CBP) is expected to be a cost-saving way for producing bioethanol (Lynd, van Zyl, McBride, & Laster, 2005), primarily because of its use of inexpensive lignocellulosic feedstocks and lower capital and operating costs due to biocatalysts that produce enzymes for the deconstruction and solubilization of feedstocks to soluble carbohydrates.

Caldicellulosiruptor bescii is a promising candidate biocatalyst for this single-step ethanol production process (Yang et al., 2016). The genus Caldicellulosiruptor are anaerobic hyperthermophiles, which can ferment a variety of organic substrates (Hamilton-Brehm et al., 2010), and produce nearly theoretical amounts of hydrogen in order to recycle redox cofactors (Bielen, Verhaar, van der Oost, & Kengen, 2013; van de Werken et al., 2008). It is capable of solubilizing lignocellulosic biomass through the activity of its suite of CAZymes (Blumer-Schuette et al., 2012, 2015; Brunecky et al., 2013), and fermenting many of the resulting soluble carbohydrates, producing primarily acetate, lactate, hydrogen, and CO₂. Some species can also produce ethanol, though this trait is not conserved across the genus. C. bescii does not natively produce ethanol as a fermentation product, but was recently engineered to produce ethanol directly from lignocellulosic biomass substrates by introducing an adhE gene, which is constitutively expressed (Chung, Cha, Guss, & Westpheling, 2014; Chung et al., 2015).

Ethanol synthesis in C. bescii relies on cofactors from the organism’s native redox system, as is the case in other CBP organisms (Biswas, Zheng, Olson, Lynd, & Guss, 2015; Li et al., 2012). These redox systems are plastic and subject to modulation through genetic modifications or by altering growth conditions. Eliminating lactate production in C. bescii increased overall hydrogen production (Cha, Chung, Elkins, Guss, & Westpheling, 2013), while eliminating the NiFe membrane-bound hydrogenase decreased ethanol yield in a strain expressing an exogenous bifunctional AdhE (Cha, Chung, & Westpheling, 2015). Another method of redox modulation by Caldicellulosiruptor is demonstrated by the closely related bacterium Caldicellulosiruptor saccharolyticus. C. saccharolyticus produces lactate upon sparging a continuously growing culture with hydrogen (Bielen et al., 2013). It was suggested that this results from the introduced hydrogen inhibits hydrogen generation by hydrogenases, resulting in NADH-driven metabolic forcing of lactate dehydrogenase activity. While Caldicellulosiruptor species rely heavily on hydrogenases and fermentation to facilitate redox balance, other metabolic components also contribute to its redox metabolism. C. bescii strains expressing exogenous alcohol dehydrogenases with different redox cofactor requirements synthesized variable amounts of ethanol when grown under similar conditions (Chung et al., 2015). These experiments demonstrate that a better understanding of cellular redox systems is needed to more effectively engineer this biocatalyst to produce ethanol more effectively.

Rex is a well characterized and conserved global redox responsive transcription factor (Ravcheev et al., 2012) that was first characterized in S. coelicolor as a global repressor able to detect and regulate gene expression in response to the intracellular [NADH]/[NAD⁺] couple (Sickmier et al., 2005). In doing so, Rex not only regulates the poise of this redox couple, but the cellular redox state in general, as evidenced by its conserved transcriptional regulation of redox genes that are not NAD(H) dependent (Ravcheev et al., 2012). Conserved Rex regulon members include genes involved in energy conversion, redox metabolism, glycolytic and fermentation genes, and NAD biosynthesis (Ravcheev et al., 2012). Examples of genes where Rex regulation is found to be taxon specific and less conserved are those of hydrogenases, heme biosynthesis, sulfate reduction (Christensen et al., 2015) and biofilm formation (Bitoun, Nguyen, Fan, Burne, & Wen, 2011), solventogenic metabolism (Zhang et al., 2014), nitrate and chloride metabolism/resistance (Carlson et al., 2015), and cytochrome biosynthesis (Larsson, Rogstam, & von Wachenfeldt, 2005).

Redox sensing and regulation by the transcriptional regulator Rex in the closely related species Caldicellulosiruptor saccharolyticus was partially inferred from a differential gene expression study of cells grown under hydrogen sparging (Bielen et al., 2013). Genes encoding elements of both the NiFe and FeFe hydrogenases were predicted to be under transcriptional control of Rex, as were the subunits of pyruvate:ferredoxin oxidoreductase. Lactate dehydrogenase is not predicted to be controlled by Rex in C. saccharolyticus (Bielen et al., 2013), though it is predicted to be controlled by Rex in other many other organisms (Ravcheev et al., 2012). Rex is predicted to be a global repressor in C. saccharolyticus, regulating expression of other regulatory elements such as a histidine kinase, a CopG family transcription factor, the iron uptake regulator Fur, as well as the rex gene itself. Other regulatory targets of Rex and action of some regulatory elements in the regulon of C. saccharolyticus remain unknown and unexplored. A consensus-binding sequence for Rex was predicted in C. saccharolyticus (Bielen et al., 2013) that is similar to predicted Rex-binding sequences identified in bacteria from other genera (Novichkov et al., 2013). The operator site in C. saccharolyticus and other organisms is an 18–20 bp palindrome sequence with the overall consensus sequence of TTGTGAANNNNTTCACAA (Ravcheev et al., 2012). The residues of the binding sequence important for Rex-DNA interaction have been shown to be its most conserved residues (Brekasis & Paget, 2003; McLaughlin et al., 2010; Pagels et al., 2010; Pei et al., 2011).

Deleting the rex gene can have dramatic effects on intracellular redox state and the production of ethanol. A strain of Clostridium acetobutylicum containing a disrupted rex gene produced more...
ethanol and butanol after 60 hr than its parent strain or a rex deletion strain complemented with the C. acetobutylicum rex gene (Wietzke & Bahl, 2012). Derepression of adhE genes, which are transcriptionally controlled by Rex in C. acetobutylicum (Wietzke & Bahl, 2012; Zhang et al., 2014), and increased NADH-dependent AdhE activity were shown to coincide with increases in ethanol and butanol production.

The goal of this study is to understand the redox metabolism in this unique and biotechnologically relevant species and genus, specifically as it pertains to ethanol synthesis, and we do so by studying the rex gene in C. bescii. A comprehensive understanding of redox metabolism in C. bescii will allow more effective engineering of redox systems to promote synthesis of ethanol at higher yield and productivity.

2 | EXPERIMENTAL PROCEDURES

2.1 | Batch growth and fermentation

C. bescii was grown in 50 ml culture volumes in sealed 135 ml serum bottles. The medium was comprised of components as described previously (Farkas et al., 2013), with maltose being used as the primary carbon source, and ammonium chloride as the primary nitrogen source. Media were prepared, adjusted to pH of 6.8, and allowed to become anaerobic overnight through dissolved oxygen exchange in an anaerobic chamber containing an environment of 5% H2, 10% CO2, and the balance N2. Cultures were grown at 75°C shaking at 200 rpm. Samples for cell growth were collected as 1 ml aliquots and measured for absorbance at OD 680. Nitrogen limited batch growth was conducted as outlined above with the only difference being the final concentration of ammonium chloride (the only source of reduced nitrogen in LOD media) of 0.467 mmol/L rather than 4.67 mmol/L as in standard LOD media.

2.2 | pH controlled fermentation

Cells were cultured in 3L Applikon Ez-Control fermenters (Applikon Biotechnology, Delft, Netherlands) in a working culture volume of 1.5 L at a growth temperature of 75°C. Maltose, resazurin, and an appropriate volume of water were added to assembled fermenters and autoclaved. Fermenters were cooled while sparging with N2 gas. Upon cooling, other media components were added as presterilized stock solutions. The media was again heated and sparged with N2 gas to ensure anaerobic conditions. Upon reaching 75°C, the pH was adjusted to 7.1 by sparging with an 80%/20% N2/CO2 gas mix. The pH was then aseptically checked using a second probe which had been calibrated with fresh pH buffers maintained at 75°C. Any differences were accounted for as the pH probe offset. Fermenters were inoculated to equivalent OD680 of 0.01–0.05 with batch grown cultures grown to mid-log phase. Stirring was maintained at 200 rpm without gas being sparged during growth, though the headspace outlet line was kept open to allow fermentation off-gasses to vent through a sterilized water trap.

2.3 | Fermentation product analysis

Samples were collected from batch serum bottles or fermenters as 1 ml aliquots and centrifuged at maximum speed in a microcentrifuge for 5 min, followed by collecting and filtering (0.22 μm) the supernatant. 250 μl of filtered supernatant was added to 1.75 μl of 2 mol/L H2SO4 and 20 μl of this mixture was injected onto a Biorad Aminex 87H column operated on a Hitachi LaChrom Ultra HPLC system (Hitachi High Technologies America, Dallas, TX). Chromatograms were collected on a Hitachi RI detector (part number L-2490).

2.4 | Mutant construction

C. bescii rex deletion mutants were constructed as described previously (Chung, Farkas, Huddleston, Olivar, & Westpheling, 2012). Strains generated for this study, necessary oligonucleotides, and plasmids used for strain generation are listed in Table 1. Briefly, an integrating suicide vector was prepared containing 1,000 bp of homology overlap to genomic sequence immediately 5′ and 3′ of the rex (ATHE_RS03255) coding sequence (Figure S1a). Plasmids were transformed into the respective genetic background, either strain JWCB005 (Chung, Cha, Farkas, & Westpheling, 2013) or strain JWCB032 (Chung et al., 2014), and transformants selected in LOD media containing no uracil to enforce prototrophic growth and plasmid integration. These cultures were subsequently plated on LOD media containing 40 μmol/L uracil (to allow for a return to strain auxotrophy) and 6 mmol/L 5-fluororotic acid (Oakwood Products Inc., Estill, South Carolina) to screen and select for the double homologous recombination event. Deletions were confirmed by PCR (Figure S1b) and Sanger sequencing of the rex gene genomic locus.

2.5 | RNA-seq analysis

Samples were collected from fermenter-grown cultures of JWCB005Δrex and its parent strain JWCB005 at early, mid, and late log phase (Figure S2a). Thereafter, 25 ml culture aliquots were harvested, centrifuged at 20,000g in a Piramoon fixed angle FiberLite rotor (ThermoScientific, Waltham, MA) for 4 min at 4°C, decanted, snap frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted by first incubating cell pellets in 250 μl of 20 mg/ml Lysozyme (Sigma Aldrich part number L-7651, St. Louis, MO) resuspended in SET buffer (50 mmol/L Tris-HCl pH 8.0, 50 mmol/L EDTA, 20% w/v Sucrose) and incubated in a dry stationary bath at 37°C for 8 min, vortexing briefly every 2 min. RNA was purified with a Qiagen RNEasy Kit according to manufacturer’s protocol (Qiagen, Hilden, Germany). RNA concentration was quantified with a Nanodrop 1000 instrument (ThermoScientific) and RNA quality was assessed via RNA Integrity Numbers (RIN) obtained with an Agilent 2100 Bioanalyzer and corresponding RNAchip (Agilent Technologies, Santa Clara, CA). Ribosomal RNA was then depleted from total RNA samples with a RiboZero rRNA Removal Kit (Illumina Inc. San Diego, CA) following manufacturer’s
instructions. Next, cDNA was synthesized from RNA depleted of ribosomal RNA with a TruSeq Stranded mRNA Library Preparation Kit (Illumina Inc) following the manufacturer’s protocol. The cDNA libraries were sequenced on an Illumina HiSeq 2500 using v4 chemistry (Illumina Inc.) and demultiplexed as a sequencing service provided by The Genomic Services Lab at HudsonAlpha Institute for Biotechnology (Huntsville, AL). Each sample library was sequenced on two different sequencing lanes and reads containing identical barcodes from each lane were combined for subsequent analysis. Reads obtained were scored for quality, trimmed, mapped, and the mapped reads counted with the corresponding functions in the CLC Genomics Workbench version 8 using default settings for genome analysis of prokaryotes. Raw read counts for each coding sequence were used as input for differential expression analysis, using the DESeq2 (Love, Huber, & Anders, 2014) package as part of the Bioconductor Suite in R.

Genes were considered differentially expressed if they displayed differential normalized log2-transformed read count abundance >0.5 or <−0.5 with a Benjamini-Hochberg adjusted p < .05.

2.6 | Metabolomic profiling

Differential metabolomic profiling was conducted on strains JWCBO32arex and JWCBO32 collected after 36 hr of batch serum-bottle growth in 50 ml culture volume of nitrogen-limiting media, as described above. Here, 50 ml replicates were collected, centrifuged, and snap frozen as described in the sample collection section of RNA-seq analysis. Additionally, following centrifugation, the supernatants were aliquoted separately, snap frozen, and stored at −80°C for metabolomic analysis. Cell biomass was pooled from three 50 ml cultures to make one replicate for intracellular metabolite analysis, as it was needed to attain sufficient signal intensity. Cell pellets and supernatants were analyzed for intracellular and extracellular metabolites, respectively, as described previously (Holwerda et al., 2014).

2.7 | Transcription factor-binding site prediction

The consensus Rex-binding sequence identified in C. saccharolyticus (Bielen et al., 2013) was used to seed a search genomic regions 300 bp upstream of every coding DNA sequence in the C. bescii genome. Binding sites were identified and scored based on similarity to this consensus-binding site, yielding 63 total putative Rex-binding sites across the C. bescii genome (Table S1). Homology scores for these sites ranged from 8.75 (least homologous) to 10.5 (most homologous).

2.8 | Rex Protein purification

Rex protein was purified by first expressing the C. bescii rex coding sequence on plasmid pTXB1 (New England Biolabs) part number N6707S, Ipswitch, MA upstream of the gyrase intein and chitin-binding domain (CBD), yielding plasmid pTSB1::rex (Table 1B). This plasmid was transformed into T7 Express E. coli cells (New England Biolabs). Cells were grown, induced and harvested according to manufacturer’s suggested instructions. Recombinant Rex (rRex) protein was purified from induced cell biomass according to protocols supplied with the IMPACT protein purification kit (New England Biolabs).

2.9 | Electromobility shift assays

Electromobility shift assays were carried out to test Rex binding to predicted operator sites listed in Table 2. Probes used were biotin-labeled 50-mers of double stranded DNA consisting of the 18 bp putative-binding site centered on the probe and surrounding genomic sequence on either side to make 50 bp. Probes were ordered as complimentary oligonucleotides, with one oligonucleotide biotin labeled (Integrated DNA Technologies, Coralville, IA). All probes were annealed in a mixture containing 3 μl of 100 μmol/L of each oligonucleotide, 3 μl of 10× polynucleotide kinase buffer (Roche part number 12579400, Roche Holding AG, Basel, Switzerland), 3 μl of 0.5 mol/L NaCl, 18 μl of DEPC treated H2O. Mixtures were heated at 96°C for 5 min in a Fisher Scientific Isotemp Stationary Bath (ThermoScientific) after which the heat block was removed from the bath and placed on a lab bench for 60 min to cool slowly. Probes were then purified using a Qiagen PCR clean-up kit (Qiagen) according to manufacturer’s instructions.

Electromobility shift assay reactions were conducted in 20 μl volumes and the following components were added in the following order: 1× EMSA-binding buffer (LightShift Chemiluminescent EMSA Kit part number 20148, ThermoScientific), 25 μg dl-dC, recombinant Rex protein in various concentrations noted on figures, and 0.1 nmol/L biotin-labeled 50 bp dsDNA probes. In reactions containing NADH or NAD+, these components were added from freshly prepared stocks kept on ice prior to adding recombinant Rex protein but after adding other components. Reactions were incubated for 30 min at room temperature after which 5 μl of EMSA loading buffer containing 15% w/v Ficoll (Sigma Aldrich, St. Louis, MO) and 0.4% w/v Orange G (Sigma Aldrich) was added to each reaction. 20 μl of the reaction mixture containing loading buffer was loaded onto a Novex 6% DNA Retardation Gel (Invitrogen # EC63652BOX, Life Technologies Corporation, Carlsbad, CA) and run in 0.5× TBE (Invitrogen # LC6675, Life Technologies Corporation) in an Invitrogen Novex Mini-Cell X Cell SureLock (Life Technologies Corporation) at 90 V for 45 min, or until Orange G from gel loading buffer had run ¾ of the length of the gel. After electrophoresis products were transferred onto Biodyne B Pre-Cut Modified Nylon 0.45 μm Membranes (Product # 77016 ThermoScientific) using the Invitrogen X Cell Blot Module (catalog # EI9051) and Invitrogen Novex Mini-Cell X Cell SureLock (Life Technologies Corporation). Transfers were carried out in 0.5× TBE at 450 mA for 45 min, with the surrounding outer minicell volume packed with ice and filled with water to cool the blot module. Biotin labeled probes were detected on transfer membranes using the ThermoScientific Chemiluminescent Nucleic Acid Detection Module Kit (part number 89880, ThermoScientific) according to manufacturer’s instructions.
3 RESULTS

3.1 rex deletion and bioinformatic prediction of the rex regulon in C. bescii

Two rex deletion mutants were generated for this study; one using strain JWCB005 (Chung et al., 2013) as the genetic parent strain, and the other, using the ethanol-producing strain JWCB032 (Chung et al., 2014) as the parent strain. Predicted promoter regions in the C. bescii genome (taken to be within the 300 bp region upstream of all predicted open reading frames) were analyzed for the presence of a putative Rex transcription factor-binding site using a C. saccharolyticus Rex consensus sequence (Bielen et al., 2013). A total of 63 possible binding sites were identified in the C. bescii genome (Table S1) and scored based on their homology to the predicted C. saccharolyticus consensus Rex-binding site sequence (Bielen et al., 2013; Novichkov et al., 2013).

3.2 Expression profiling of JWCB005Δrex and selecting transcription factor-binding sites for in vitro verification

Differential transcript expression was conducted with strain JWCB005Δrex and its parent strain JWCB005 to identify genes and transcriptional units under Rex control. Samples were collected at early, mid, and late log phase, (Figure S2a). An average of 83.2% of RNA-seq reads that passed quality assurance aligned uniquely to the C. bescii genome (S.D. 1.3%), with an average per-sample genome coverage of 276X (S.D. 28). Increased expression during at least one timepoint in JWCB005Δrex relative to its parent strain was observed for 15 genes, while 9 showed decreased expression (Table S2). A summary of genes identified as being likely members of the Rex regulon in C. bescii, identified through differential expression, previous, as well as our own de novo-binding site predictions, and their corresponding putative Rex-binding sites were tested by electromobility shift assay (Table 2).

### TABLE 1 Primers, plasmids, and C. bescii strains generated and/or used in this study

| (A) | Primer name | Sequence |
|-----|-------------|----------|
| pDCW88 gib assy backbone fwd | gtgcactctgcgccgctc |
| pDCW88 gib assy backbone rev | gttaaccagctccaac |
| pDCW88_ate_0654_up_fwd | tccaatgatcgaagttaggctggtggtaccatatcttcaattttgctccagcag |
| pDCW88_ate_0654_up_rev | ttacataacgcattcatttcacctcaagtccttttctcccccttatcttttg |
| pDCW88_ate_0654_down_fwd | gaccttggaggttaatgatgct |
| pDCW88_ate_0654_down_rev | gttttctgccatctgagtcagtgccacactttctaatattctgcaccaag |
| upstm 5′ flank fwd athe_0654_P3 | agaatattgaagcgccgaac |
| dnstm 3′ flank rev ath_0654_P3 | gtggaaaaatcccccagaa |
| internal fwd athe_0654_P3 | ggttttgctgcaagggata |
| internal rev ath_0654_P3 | accctaatccccactctt |
| 3′ flank rev seq ath_0654_P3 | ttgcagagtttcgtaaga |

| (B) | Plasmid | Description | Reference |
|-----|---------|-------------|-----------|
| pETE01 | Non-replicating suicide vector used to generate strains JWCB005Δrex and JWCB032Δrex | This study |
| pTXB1::rex | IPTG-inducible expression vector used to express recombinant Rex protein in E. coli T7 Express | This study |

| (C) | Strains | Description | Genotype | Reference |
|-----|---------|-------------|----------|-----------|
| JWCB005 | Genetic background strain used to generate JWCB005Δrex, wildtype ldh locus | ΔpyrFA (ura<sup>+</sup>/5-FOA<sup>+</sup>) | Farkas et al. (2013) |
| JWCB005 Δrex | Markerless rex deletion using strain JWCB005 as genetic parent | ΔpyrFA Δrex (ura<sup>+</sup>/5-FOA<sup>+</sup>) | This study |
| JWCB032 | Genetic background strain used to generate JWCB032Δrex, ldh locus disrupted by IS5be4, expressing genomically bifunctional alcohol dehydrogenase (AdhE) from C. thermocellum | ΔpyrFA ldh:::IS5be4 Δcbel::Ps-layer Cthe-AdhE (ura<sup>+</sup>/5-FOA<sup>+</sup>) | Hamilton-Brehm et al. (2010) |
| JWCB032 Δrex | Markerless rex deletion using strain JWCB032 as genetic parent | ΔpyrFA Δrex ldh:::IS5be4 Δcbel::Ps-layer Cthe-AdhE (ura<sup>+</sup>/5-FOA<sup>+</sup>) | This study |
3.3 | Electromobility shift assays to test rex binding to predicted C. bescii binding sites

A series of operator sites that displayed an in vitro Kₐ between 10 and 50 nmol/L were identified, similar to other in vitro Rex-binding studies in other Gram-positive organisms (Brekas & Paget, 2003; Wang et al., 2008; Zhang et al., 2014). Sequence specificity and NADH cofactor-binding specificity of in vitro binding reactions were also verified (Figures S4, S5, and S). Furthermore, operator sites giving these Kₐ values are those located in the promoter regions of genes shown to be conserved members of the Rex regulon (Ravcheev et al., 2012). Four unique Caldicellulosiruptor Rex-binding sites were identified in the promoter regions upstream of the genes ATHE_RS00825, ATHE_RS04105, ATHE_RS04720, and ATHE_RS10680. These genes comprise regulon members for Rex in Caldicellulosiruptor not previously identified (Figures 1–5).

3.4 | Ethanol productivity of a rex deletion in an ethanol producing strain of C. bescii

To test the direct impact of the Rex protein on ethanol production in C. bescii, we deleted the rex gene in an ethanol producing strain, JWCB032 (Chung et al., 2014). JWCB032Δrex showed no differences in growth profile or fermentation products relative to its parent after 48 hr when grown in LOD media (Figure S6). However, phenotypic differences were observed when these two strains were grown in LOD media augmented with 1/10th the typical amount of ammonium chloride (0.467 mmol/L), the only source of soluble reduced nitrogen in LOD media (Farkas et al., 2013), making this a nitrogen-limited growth condition. C. bescii actively grows for only 10–12 hr when grown in this condition (Figure 6a). Both strains grew to similar turbidity under this growth condition, with the rex mutant exhibiting a lag phase about 5 hr longer than its parent strain (Figure 6). Differences in fermentation product profile appear after active growth ceases (>36 hr, Figure 6b). The rex knockout strain produced 54% more ethanol after 36 hr of fermentation than its parent strain (Figure 6), accounting for a 0.16 mmol/L difference in final ethanol concentration between the two strains. The two strains produced similar amounts of acetate after 36 hr of fermentation. The relative difference in the amount of ethanol produced in JWCB032Δrex relative to JWCB032 increased through 60 hr of fermentation.

3.5 | Metabolite profile differences observed in ethanol producing rex-deficient C. bescii

To investigate ethanol production differences between the two strains under nitrogen-limiting conditions, intracellular, and extracellular metabolomic profiles were generated after 36 hr of fermentation (Figure 7, Table S3), when differences in ethanol concentrations were previously observed to be prominent (Figure 6). Succinate and 2-oxoglutarate, two TCA cycle metabolites, show a shift in TCA cycle carbon flux toward succinate production in JWCB032Δrex. The relative intracellular abundance of 2-oxoglutarate in JWCB032Δrex was only 18% of that of strain JWCB032, while the corresponding relative intracellular concentrations of succinate were 125% higher in JWCB032Δrex. Lactate, pyruvate, glycerol/glycerol-3P, and hexadecanoate were all found to be at least 124% more abundant in JWCB032Δrex. The relative abundance of many amino acids was increased either intracellularly or in the supernatant in JWCB032Δrex. Mesaconate and citramalate were found to be at least 138% more abundant in JWCB032Δrex in both the supernatant and intracellularly, while oxalomalate was found to be less abundant in both. Cystine was found in decreased abundance in JWCB032Δrex, both intracellularly (37% of parent strain) and in the supernatant (93% of parent strain).

4 | DISCUSSION

4.1 | Expanding the rex regulon in C. bescii

The Rex regulon of the close C. bescii relative, Caldicellulosiruptor saccharolyticus, has been curated in the RegPrecise database (Novichkov et al., 2013). RegPrecise predicts six Rex-binding sites in C. saccharolyticus. Two of these sites are located upstream of adhA genes (C SAC_0622 and C SAC_0407) of which C. bescii does not have a homolog of. Homologs of the remaining four predicted Rex operator sites predicted in C. saccharolyticus were found on the C. bescii genome (Table 2). A study of gene expression in C. saccharolyticus under hydrogen sparging (Bielen et al., 2013) found evidence to extend the putative Rex regulon of C. saccharolyticus beyond those genes predicted in RegPrecise. Our study validates Rex regulatory control of these previously predicted regulon members, as well as novel members of the Rex regulon not previously predicted; the HydG hydrogenase maturation factor collocated in a putative operon with a CopG family transcription factor, the ‘XOR’ gene (a poorly annotated oxidoreductase) (Scott et al., 2015), an unannotated highly expressed oxidoreductase gene, and a class V aminotransferase.

HydG is responsible for synthesizing a di-iron precursor to the H-cluster active site of the FeFe hydrogenase (Kuchenreuther et al., 2014). As the presence or absence of HydG protein can effectively regulate assembly of functional FeFe hydrogenase by modulating correct active site assembly (Biswas et al., 2015), and the FeFe hydrogenase is a main hydrogen generation route in C. bescii (Cha et al., 2015), its regulation by Rex is consistent and expected. The regulon of this CopG transcription factor remains unknown and unpredicted.

Rex regulation of the ‘XOR’ gene is also to be expected given its presumed importance to redox metabolism in C. bescii (Scott et al., 2015). While Rex is only known to sense the redox potential of the NADH/NAD⁺ redox couple within the cell, it regulates redox metabolic reactions whose cofactors stretch beyond just this couple, exemplified in C. bescii by Rex regulation of the ferredoxin-dependent NiFe membrane bound hydrogenase (Figure 2) and of a likely ferredoxin-dependent pyruvate:ferredoxin oxidoreductase (Figure 3). While the specific function of this gene remains unknown, its relatively high expression level, unique reliance on tungsten, and coordinated expression with tungsten assimilation
| Locus tag downstream of operator site | Predicted transcription unit | Distance from ATG (bp) | RegPrecise (Novichkov et al., 2013) Predicted Regulon | Predicted Regulon in (Bielen et al., 2013) | Site Homology Prediction Score (This study) | log$_2$(JWCB005Δrex/JWCB005) of downstream gene | Early Log Phase | Mid Log Phase | Late Log Phase |
|-------------------------------------|------------------------------|------------------------|-----------------------------------------------------|---------------------------------------------|-----------------------------------------------|-------------------------------------------------|----------------|--------------|--------------|
| ATHE_RS00825, Athe_0168             | 2 genes; CopG family transcriptional regulator, HydG hydrogenase maturation protein | 181 | X | X | 10.5 | -0.21 | -0.23 | -0.27 |
| ATHE_RS03255, Athe_0654             | 1 gene; Rex | 41 | X | X | 9.25 | N/A | N/A | N/A |
| ATHE_RS04105, Athe_0820             | 2 genes, Ferredoxin, ‘XOR’ oxidoreductase protein (Scott et al., 2015) | 148 | | | 9.5 | 0.86 | 0.60 | 0.75 |
| ATHE_RS04125, Athe_0824             | 3 genes; tungsten transport system (Scott et al., 2015) | 274 | | | 8.75 | 0.47 | 0.38 | 0.77 |
| ATHE_RS04390, Athe_0874             | 4 genes; subunits of pyruvate/ketoisovalerate:ferredoxin oxidoreductase | 112 | X | | 9.5 | -0.28 | -0.73 | -0.62 |
| ATHE_RS04720, Athe_0942             | 2 genes; hypothetical protein, hypothetical protein | 40 | | | 10 | -1.46 | -1.42 | -0.83 |
| ATHE_RS05415, Athe_1082             | 19 genes; Ech hydrogenase system | 147 | X | X | 9 | -0.43 | -0.80 | -1.13 |
| ATHE_RS06475, Athe_1295             | 5 genes; Hyd hydrogenase system | 39 | X | X | 10 | -0.13 | -0.35 | -0.38 |
| ATHE_RS10680, Athe_2126             | 3 genes; class V aminotransferase, phosphoglycerate dehydrogenase (NADH), hypothetical protein | 88 | | | 8.75 | 0.59 | 0.63 | 0.57 |
| ATHE_RS11210, Athe_2226             | 1 gene; pyruvate carboxyltransferase (KEGG) or 2-isopropylmalate synthase LeuA (RefSeq) | 169 | X | | 8.75 | 0.08 | 0.12 | 0.22 |

Sites were chosen from homologous sites identified in the RegPrecise (Novichkov et al., 2013) curated regulon, previously inferred Rex-binding sites in C. saccharolyticus (Bielen et al., 2013), and observed differential expression between JWCB005Δrex and its parent strain (JWCB005). Gray shading indicates nonsignificant values. Black values indicate values which are significant (Benjamini-Hochburg adjusted $p < 0.05$). Putatively regulated transcriptional unit operon structure are reported as predicted in the DOOR database (Mao et al., 2014) and previous literature (Scott et al., 2015; van de Werken et al., 2008).
genes and a ferredoxin suggest its importance to redox metabolism in C. bescii.

Rex regulatory control of a class V aminotransferase in C. bescii is not well understood. A serine-pyruvate aminotransferase was identified as part of the Rex regulon of five genomes from Thermotogales, constituting an NADH-dependent step of a serine utilization pathway (Ravcheev et al., 2012). This Rex regulated class V aminotransferase in C. bescii is annotated as an alanine-glyoxylate aminotransferase and no reference is given to its redox dependence. This gene is also collocated in a putative transcriptional unit with an NADH dependent 3-phosphoglycerate dehydrogenase, also a biochemical step in serine biosynthesis.

4.2 | Ethanol-producing rex-deficient C. bescii produces more ethanol under nitrogen-limiting conditions which extends fermentation

A rex deletion strain was generated, using C. bescii strain JWCB032 (Chung et al., 2014) as the parent to investigate the effect Rex may have on ethanol production and overall redox metabolism. Relative differences in ethanol synthesis were observed in a rex-deficient mutant strain when the cells were grown in nitrogen-limiting conditions (Figure 6), though not in replete media, and only after 36 hr of fermentation. Under replete conditions, the pH of batch cultures of C. bescii drop to ~4.5 from an initial pH of 7.2 (data not shown) due mainly to the production of acetic acid. Growth and fermentation are not observed at or below these pH values (data not shown). Growing the cells in nitrogen-limiting conditions restricts active growth, the total amount of cell biomass synthesized, and acts to limit the total amount of acetate that is generated (as seen by comparing nitrogen limited growth and fermentation products in Figure 6 and replete fermentation product production in Figure S6), allowing fermentation to continue long after active growth has ceased. A shift was observed in whole-cell redox state in C. saccharolyticus as it entered the stationary phase, as indicated by an increase in lactate production and lower response when harvested cells were subjected to a poised amperometric cell (Kostesha, Willquist, Emmeus, & van Niel, 2011). This indicates redox-mediated end-product shifting is occurring as cells enter stationary phase, likely favorable conditions for ethanol production as the exogenous AdhE of JWCB032 and native lactate dehydrogenase both rely on NADH to generate their respective products. C. cellulolyticum grown in nitrogen-limited chemostat culture similarly showed an increase in fermentative flux toward ethanol as dilution rates increased (Desvaux & Petitdemange, 2001). Ethanol generation by heterologously expressed AdhE protein expressed in strain JWCB032 has been shown to be dependent on redox conditions in the native host. Perturbing redox metabolism in C. thermocellum, the native host of this AdhE enzyme, either through genetic modification or altering growth conditions, has predictably resulted in altered levels of the formation of ethanol and other reduced fermentation products (Biswas et al., 2015; Brown et al., 2011; Rydzak, Levin, Cicik, & Sparling, 2011; Sander et al., 2015). It is likely this AdhE enzyme is similarly sensitive to redox conditions when expressed in C. bescii.

Another possible explanation for elevated levels of ethanol in JWCB032Δrex under nitrogen limitation would be an interaction between the rex regulator and nitrogen metabolism in C. bescii, which could be augmented in the JWCB032Δrex strain deficient in this regulator. Metabolic and expression coupling between nitrogen metabolism and ethanol production has been studied in mutant strains of C. thermocellum (Rydzak et al., 2017), though no regulatory link involving Rex was found. No known link between nitrogen metabolism and ethanol production involving Rex in C. bescii is known to exist either.
To investigate why JWCB032Δrex produced more ethanol than its parent strain, intracellular, and extracellular metabolomics was conducted on this strain and its parent, JWCB032. Most metabolites found in increased abundance in strain JWCB032Δrex require reduction reactions (with reductant being provided by NADH and NADPH in most cases) for their synthesis. Apart from the glyceral and glycerol-3P, inferred metabolic pathways (Kanehisa, Sato, Kawashima, Furumichi, & Tanabe, 2016) responsible for the synthesis of metabolites found in differential abundance originate at the pyruvate metabolic pathway node (Figure 7). The accumulation of pyruvate and the redox-state reduction of intracellular redox pools may support the synthesis of overflow metabolites.

4.3 | Redox buffering systems

Increased concentrations of components of two known redox buffer systems were observed in JWCB032Δrex cells; the cysteine–cystine couple (Banerjee, 2012) and the 2-oxoglutarate-glutamate couple (Ballester-Tomás, Randez-Gil, Pérez-Torrado, & Prieto, 2015; Mailloux et al., 2009). The reactions of both couples involve the interchange of one molecule of NAD(P)H and 2 electrons. The oxidized component of each couple was found in decreased concentration in JWCB032Δrex, suggesting that these redox buffer systems are responding to more reduced redox conditions relative to those of strain JWCB032 by reducing the pools of these molecules. Cysteine is known to be one of the most reducing of the known cellular redox buffer systems (Banerjee, 2012). Free cysteine has been shown to reduce iron in E. coli, generating hydroxyl radicals and causing DNA damage, and this may be an evolutionary reason intracellular free cysteine levels are kept low in cells (Park & Imlay, 2003). Components of other cellular redox buffer systems were not detected in our metabolomic study of JWCB032 and JWCB032Δrex. C. bescii redox conditions may be more readily augmented using the cysteine–cystine system rather than other redox buffering systems.

4.4 | Differentially abundant metabolites in JWCB032Δrex

Metabolites involved in glycerol metabolism, biosynthesis of the fatty acid hexadecanoate, five amino acids (isoleucine, phenylalanine, alanine, valine and leucine), TCA cycle metabolites, ethanol, and acetate were found in increased abundance in JWCB032Δrex.
FIGURE 3 Proposed models of Rex repression supported by electromobility shift assays of binding sites upstream of central glycolytic genes. These results suggest Rex represses expression of ferredoxin-dependent pyruvate/ketoisovalerate oxidoreductase (Solid red line). Rex transcriptional control of pyruvate carboxyltransferase (ATHE_RS11210, also annotated as oxaloacetate decarboxylase), though remains doubtful because of the relatively high $K_d$ observed in vitro (dashed red line).

FIGURE 4 Electromobility shift assays of other predicted Rex-binding sites whose role in redox metabolism, and C. bescii metabolism in general, is not well understood. Rex regulates expression of ATHE_RS10860, a class V aminotransferase as well as ATHE_RS04720, annotated as a hypothetical protein.

FIGURE 5 Electromobility shift assay showing DNA binding by Rex is disrupted by NADH and is sensitive to NADH/NAD+ ratio across cofactor pool concentrations of 1–100 μmol/L.
As the genes responsible for biosynthesis of these metabolites were not differentially expressed in strain JWCB005Δrex, we hypothesize that these differences result from metabolite-driven flux. The metabolites glycerate, glycerol, and glycerol 3-phosphate were found in relatively increased abundance in strain JWCB032Δrex, and two reactions involved in the synthesis of these metabolites (glyceraldehyde dehydrogenase and glycerol dehydrogenase) are redox dependent. There are no genes annotated in the C. bescii KEGG database entry (Kanehisa et al., 2016) that encode catalytic enzymes for these reactions, nor are there any genes annotated with these functions in the recently re-annotated RefSeq annotations for the C. bescii DSM 6725 genome, though the presence of these molecules indicates these functions must be present in vivo in C. bescii. It is worth noting that C. bescii cannot grow in media containing glycerol as a sole source of carbon (Hamilton-Brehm et al., 2010). Increased abundance of amino acids, particularly branched chain amino acids, was also observed as products of overflow metabolism of C. thermocellum (Biswas et al., 2017; Holwerda et al., 2014) in response to genetically induced redox perturbations and increased substrate loading. Metabolic flux of the TCA cycle and associated reactions was also redistributed toward reductive reactions, resulting in increased supernatant concentrations of succinate and glutamate and decreased supernatant and intracellular concentrations of oxalomalate and 2-oxoglutarate. Succinate production was increased 60% in E. coli by altering redox metabolism (Singh, Cher Soh, Hatzimanikatis, & Gill, 2011), highlighting the importance intracellular redox can have on the synthesis of succinate and TCA cycle flux.

According to the KEGG functional annotation (Kanehisa et al., 2016) of the C. bescii genome, metabolites whose synthesis do not require redox reactions in reaction steps unique to their synthesis are mesaconate, citramalate, oxalomalate, and acetate. Acetate and oxalomalate are competing pathways to metabolites whose synthesis pathways do depend on redox reactions, and the relatively decreased amount of these metabolites may be due to redox-driven flux through these competing pathways. Mesaconate can be synthesized from pyruvate or glutamate. Citramalate and mesaconate production are sequential metabolic steps, and because citramalate is also found in increased abundance, mesaconate is likely synthesized from pyruvate rather than from glutamate. KEGG does not annotate/assign genes for mesaconate synthesis from pyruvate or glutamate in C. bescii, though the most recent RefSeq annotation of the C. bescii DSM 6725 genome does contain an annotation for a gene encoding a citramalate synthase (ATHE_RS02505). No gene encoding a mesaconate hydratase, the enzyme synthesizing mesaconate from citramalate, could be identified. Neither of the two genes necessary for mesaconate synthesis from glutamate are annotated and protein BLAST homology searches for methylaspartate ammonia-lyase from E. coli and Aspergillus oryzae returned no genes with significant similarity. This further suggests mesaconate is being generated from citramalate as an intermediate and as an overflow metabolism product resulting from the accumulation of pyruvate.

### 4.5 Intracellular redox conditions and pyruvate accumulation possibly driving metabolite differences

Many reactions synthesizing differentially abundant metabolites are [NADH]/[NAD+] dependent, and the observation of cellular redox buffer systems being active, further supports the hypothesis that metabolite differences observed in JWCB032Δrex stem, in part, from redox-driven flux differences. Redox-driven overflow metabolism is observed in two other CBP organisms, *Clostridium thermocellum* (Holwerda et al., 2014) and *Clostridium*...
cellulolyticum (Guedon, Payot, Desvaux, & Petitdemange, 1999). Excess-free amino acids are found in the supernatant of strains of C. thermocellum whose redox metabolism has been perturbed (Holwerda et al., 2014), and strains of C. cellulolyticum grown at high dilution rates (Guedon et al., 1999). Upon being challenged with these increased reductive loads, pyruvate is found in the supernatant of both species and both species synthesize lactate (Guedon et al., 1999; Holwerda et al., 2014). C. cellulolyticum also shunts carbon at an earlier glycolytic node (Glucose 1-phosphate → Glucose 6-phosphate) toward glycogen and exopolysaccharide synthesis in response to increasing substrate loads (Guedon, Desvaux, & Petitdemange, 2000). Pyruvate accumulation and subsequent overflow metabolism has been observed and studied in detail in C. thermocellum (Deng et al., 2013; Holwerda et al., 2014; Olson et al., 2017; Thompson et al., 2015). Accumulation of formate and hydrogen in C. thermocellum collectively restrict the reoxidation of ferredoxin and limit its availability for pyruvate:ferredoxin oxidoreductase-enabled conversion of pyruvate to acetyl-CoA, causing accumulation of pyruvate and pyruvate-derived overflow metabolism products (Holwerda et al., 2014; Thompson & Trinh, 2017; Thompson et al., 2015). C. cellulolyticum growth was shown to be inhibited by high concentrations of NADH, which inhibits glyceraldehyde 3-phosphate dehydrogenase activity and limits glycolytic flux (Payot, Guedon, Cailliez, Gelhaye, & Petitdemange, 1998). Substantial overflow metabolism and increased ethanol synthesis was observed at relatively lower [NADH]/[NAD⁺] ratios in C. cellulolyticum (Guedon et al., 1999), as to where strains of C. thermocellum demonstrating the most overflow metabolism and highest ethanol yields generally exhibit relatively higher
increase in final titer) when fermentation continued for 36 hr parts, suggesting the elimination of the known redox buffering systems toward their reduced counter differential abundance of reduced products and the shift of two strain heterologously expressing a bifunctional alcohol dehydrogenase gene produced 54% more ethanol (a 0.16 mmol/L tion of the Rex protein, remains unknown. A rex deletion mutant strain heterologously expressing a bifunctional alcohol dehydrogenase gene produced 54% more ethanol (a 0.16 mmol/L increase in final titer) when fermentation continued for 36 hr (while limited for nitrogen). Metabolomic profiling shows differential abundance of reduced products and the shift of two known redox buffering systems toward their reduced counterparts, suggesting the elimination of the rex gene leads to a more reduced intracellular redox environment in the stationary phase that in turn drives increased production of ethanol and other overflow metabolites.

5 | CONCLUSIONS

In studying the rex gene in C. bescii, we have found novel members of the Rex regulon not previously described. These include the ‘XOR’ gene (Scott et al., 2015), an oxidoreductase gene that is highly expressed in C. bescii, a class V aminotransferase (ATHE_RS10680), a hypothetical protein (ATHE_RS04720), and the hydG gene contained in a putative transcriptional unit along with a CopG family transcription factor. The functional contribution of these genes to redox homeostasis, the primary function of the Rex protein, remains unknown. A rex deletion mutant strain heterologously expressing a bifunctional alcohol dehydrogenase gene produced 54% more ethanol (a 0.16 mmol/L increase in final titer) when fermentation continued for 36 hr (while limited for nitrogen). Metabolomic profiling shows differential abundance of reduced products and the shift of two known redox buffering systems toward their reduced counterparts, suggesting the elimination of the rex gene leads to a more reduced intracellular redox environment in the stationary phase that in turn drives increased production of ethanol and other overflow metabolites.

ACCESSION NUMBERS

Raw reads, processed data and experiment metadata were submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GSE102041.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information section at the end of the article.

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