# Metabolic Response of *Clostridium ljungdahlii* to Oxygen Exposure

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## ABSTRACT

*Clostridium ljungdahlii* is an important synthesis gas fermenting bacterium used in the biofuels industry, and a preliminary investigation showed that it has some tolerance to oxygen when cultured in rich mixotrophic medium. Batch cultures not only continue to grow and consume $H_2$, CO, and fructose after 8% $O_2$ exposure, but fermentation product analysis revealed an increase in ethanol concentration and decreased acetate concentration compared to non-oxygen exposed cultures. In this study, the mechanisms for higher ethanol production and oxygen/ROS detoxification were identified using a combination of fermentation, RNAseq differential expression, and enzyme activity analyses. Results indicate that the higher ethanol and lower acetate concentration were due to the carboxylic acid reductase activity of a more highly expressed predicted aldehyde oxidoreductase (CLJU_c24130) and that *C. ljungdahlii*'s primary defense upon oxygen exposure is a predicted rubrerythrin (CLJU_c39340). The metabolic responses of higher ethanol production and oxygen/ROS detoxification were found to be linked by cofactor management, substrate and energy metabolism. This study contributes new insights into the physiology and metabolism of *C. ljungdahlii* and provides new genetic targets to generate *C. ljungdahlii* strains that produce more ethanol and are more tolerant to syngas contaminants.


**Keywords:** Aldehyde oxidoreductase, *Clostridium ljungdahlii*, Ethanol, Oxygen, Reactive oxygen species; Rubrerythrin, Synthesis gas fermentation

54

55 **INTRODUCTION**

56 *Clostridium ljungdahlii* is an anaerobic, motile, endospore-forming, gram-positive, rod-
57 shaped, acetogenic bacterium isolated from chicken yard waste, and has application as a
58 biocatalyst to transform syngas components (CO, $CO_2$, $H_2$) into more valuable chemicals (5, 51,
59 57, 92). It was the first bacterium discovered to metabolize these syngas components and
60 produce ethanol with acetate as its primary fermentation product (5, 92). To reduce the amount
61 of acetate and increase the amount of ethanol produced by *C. ljungdahlii* for its use in industrial
62 solvent production, different reactor designs and agitation parameters, varied syngas component
63 concentrations, increased gas flow rates and pressures, reduced nitrogen sources, addition of
64 reducing agents to media, adjustment of growth media pH, and addition of nanoparticles have all
65 been evaluated (5, 15, 16, 46-50, 66, 74, 97, 106). Some of these efforts have reported improved
66 ethanol to acetate product ratios from 1:20 to 2:1 in batch cultures and from 1:1 to 21:1 for
67 cultures grown in continuously stirred reactors (43, 47, 48). More recent sequencing and genetic
68 modification techniques have greatly enhanced our understanding of *C. ljungdahlii*'s metabolism
69 and increased production of ethanol as well as enabled production of other chemicals (e.g.
70 acetone, butyrate, butanol) (4, 51, 52, 57, 80, 91, 96).

71 Despite these advancements, for *C. ljungdahlii* to be effectively used for industrial syngas
72 transformation, some of its catalytic limitations related to gaseous headspace composition still
73 require evaluation. Though steel mill waste gas was recently used as a sole carbon and energy
74 source in a *C. ljungdahlii* fermentation, artificial syngas is commonly used in research
75 experiments to limit the variability of results (52). Artificial syngas does not typically contain
76 contaminants common to industrial syngas streams such as oxygen ($O_2$), sulphurous species

(COS, $SO_2$, $H_2S$), nitrogenous species such as (HCN, $NH_3$, NOx), methane ($CH_4$), and tars (18, 104). Several studies have been performed to evaluate the effect of these syngas contaminants on *Clostridia sp.*; however, inhibitory compounds in syngas have slowed the progress of companies moving into the commercial phase of syngas-based fermentation product development (http://www.biofuelsdigest.com/bdigest/2014/09/05/on-the-mend-why-ineos-bio-isnt-reporting-much-ethanol-production/) (30-32).

Researchers have observed that concentrations up to 2.7% $H_2S$ do not significantly affect substrate ($H_2$ and CO) uptake by *C. ljungdahlii*, and growth is maintained with concentrations as high as 5.2% (46, 48). In a related bacterium, *C. carboxidivorans* $P7^T$, concentrations of $NO_x$ above 0.0040% in syngas were found to inhibit growth and non-competitively inhibit hydrogenase activity (2, 21). Though NOx inhibition resulted in higher ethanol production by *C. carboxidivorans* $P7^T$, hydrogenase activity was inhibited, reducing available carbon for product formation since electrons came from CO rather than $H_2$ (2, 21). $NH_3$ is a nitrogen source for syngas fermenting bacteria, but in high concentrations from a continuous syngas feed it can inhibit cell growth and decrease acetate to ethanol conversion of related bacterium *C. ragsdalei* (P11) (104). In chemostat experiments with *C. carboxidivorans* $P7^T$, tars were shown to promote cell dormancy but increase ethanol to acetate production ratios (1). With *C. ragsdalei* (P11), up to 5% $CH_4$ in syngas did not affect product formation or cell growth (100). Oxygen is also a common contaminant of syngas that can have significant impact on microbial catalyzed syngas fermentation; however, an understanding of how the clostridial catalysts manage oxygen and reactive oxygen species has not been well examined to date (18).

Several enzymes of the Wood-Ljungdahl pathway, including hydrogenase and carbon monoxide dehydrogenase (CODH), responsible for syngas metabolism are sensitive to oxygen



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(3, 11, 19, 24, 34, 37, 60, 62, 67, 73, 77-79). Pyruvate:ferredoxin oxidoreductase (PFOR), pyruvate formate lyase (PFL) and its activating enzyme (enzymes involved in sugar metabolism) are also oxygen labile in a variety of microbes (6, 9, 12, 35, 58, 69, 105). Therefore, acetogens in general which possess some or all of these oxygen labile enzymes have traditionally been classified as strict anaerobes; nevertheless, they have been isolated from different aerobic or microaerobic environments (23, 25, 89). It has been demonstrated that many of these acetogens are equipped with an assortment of oxidative stress enzymes and some can even reduce oxygen by other mechanisms (e.g. superoxide reductase, peroxidase) (10, 20, 39, 41, 42, 54, 86). In addition to tolerating varying amounts of oxygen, it has been suggested that exposing acetogens to microaerobic conditions triggers a shift in electron flow towards more reduced products such as ethanol, lactate, $H_2$, and/or $NH_4^+$ production instead of acetate formation (22, 23, 54). *C. ljungdahlii* was previously shown to grow well and co-ferment fructose and artificial syngas in a complex medium (15, 16, 95). Upon exposure to low concentrations of oxygen (<10%) in the headspace of batch cultures at early log phase, *C. ljungdahlii* continued to grow and co-ferment fructose and artificial synthesis gas components while showing an increase in ethanol to acetate ratios (94). These initial experiments suggested *C. ljungdahlii* had the mechanisms to handle oxygen contaminants and positively affect solvent production, but the response was not fully elucidated. Therefore, the objectives of this study were to characterize *C. ljungdahlii*'s transcriptional, metabolic, and physiological response to oxygen exposure when grown in rich mixotrophic medium with a particular focus on the effect oxygen had on ethanol and acetate formation.

## MATERIALS AND METHODS



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**Media and growth conditions**

*C. ljungdahlii* (ATCC 55383), was obtained from the American Type Culture Collection and was cultured in Modified Reinforced Clostridial Medium (mRCM) supplemented with 5 g/L fructose (mRCMf) (15, 16). The artificial syngas composition (20% CO, 20% $CO_2$, 10% $H_2$ with $N_2$ balance) that was injected into the headspace (110 ml) of batch cultures was selected based on the approximate theoretical composition of a gas stream from biomass gasification with air as the fumigator.

The medium was prepared and dispensed into 160 ml serum bottles (final volume including reducing agents and inoculum was 50 ml/bottle). Reducing agents (2.5% w/v cysteine-HCl and 2.5% w/v sodium sulfide) were added to better ensure anaerobicity of growth media. However, in some experiments, reducing agents were omitted in an effort to observe their effect on oxygen removal, protein production (as observed on SDS-PAGE gel), and benzyl viologen reduction by *C. ljungdahlii* cell-free cell extract. Bottles were capped with butyl rubber stoppers (Bellco, Vineland, NJ) and aluminum seals, connected to a vacuum manifold using a needle and a 0.22 μm filter and made anaerobic by cycling three times (30 s cycles) between 1) vacuum headspace evacuation and 2) sparging with artificial syngas filtered through heated copper pellets. The bottles were then autoclaved for 30 min (121 °C, 15 psig). After bottles cooled to room temperature, excess pressure was released from the bottles by inserting a needle connected to a 0.22 μm filter and a hose with its end immersed in a water trap to achieve a condition of atmospheric pressure. Culture bottles were initially inoculated with an aliquot from freezer stocks (10% v/v) and incubated with shaking at 100 rpm at 37°C. After 24 h, culture aliquots (5% v/v) were transferred to fresh media and incubated (100 rpm, 37°C). After 12 h of growth, experiments were initiated with a second transfer of actively growing cells (5% v/v) into fresh



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146 media and incubated (100 rpm, 37°C). For the oxygen exposure experiments, after 12 h of
147 culture incubation, 8% (by headspace volume) $O_2$ was injected by syringe.

148 **Gas and liquid product analysis**

149 Liquid samples for ethanol and acetate were taken at 12, 14, 24, 36, 48, and 72 h time
150 points from the *C. ljungdahlii* cultures and stored (-20ºC) prior to analysis. Gas samples for CO,
151 $H_2$, and $CO_2$ and liquid samples for fructose were taken at 0, 24, 36, 42, 54, and 66 h. Fructose
152 samples were also stored (-20ºC) prior to analysis but gas was analyzed within 1 h of sampling.
153 Gas and liquid product analysis results were reported as an average of six replicates completed as
154 triplicates of two biological repeats.

155 Liquid samples (500 µl) for ethanol and acetate analysis were prepared by adding 125 µl
156 25% m-phosphoric acid and centrifuging at 14,000 x *g* for 10 min at room temperature. The
157 sample supernatants were analyzed by gas chromatography using an Agilent 7890A gas
158 chromatograph containing a 0.25 µm J & W DB-FFAP column (30 m x 0.32 mm i.d.) and a
159 flame ionization detector. Argon was used as the carrier gas at a flow rate of 30 ml/min. The
160 injector and detector temperatures were 250 °C and the oven temperature 160 °C.

161 Headspace gas samples were collected using a 5 ml gas-tight syringe with sample lock
162 (SGE, Ringwood, Australia). The samples were analyzed by gas chromatography using an
163 Agilent 7890A gas chromatograph containing a 13823 molsieve 5 Å zeolite molecular sieve
164 packed stainless steel column (6 ft x 1/8 in. i.d. Supelco) and a thermal conductivity detector.
165 Argon was used as the carrier gas at a flow rate of 30 ml/min. The injector and detector
166 temperatures were 150 °C and 250 °C respectively, and the oven temperature was 160 °C.

167 The amount of fructose present in the culture media was determined by high-performance
168 liquid chromatography. Liquid samples (600 µl) were centrifuged (10 min, 14,000 x *g*, room

temperature). Supernatant was filtered through a 0.22 µm syringe filter and analyzed using a Shimadzu LC20 liquid chromatograph with a HPX-87H column (65 °C) and refractive index detector. Sulfuric acid (5 mM) was used as the eluent at a rate of 0.6 ml/min.

Dissolved oxygen (DO) and dissolved $CO_2$ species (including bicarbonate in the liquid phase) were also quantified during batch culture fermentations. DO in the 0 and 8% $O_2$ exposed cultures was measured with a NeoFox System (Ocean Optics, Dunedin, FL, USA) as per the manufacturer's instructions at 14, 30, and 36 h to determine if oxygen in the medium was being reduced over time. To take the measurements, bottles were opened in a fume hood, the NeoFox probe was immediately lowered to the bottom of the bottles, and readings were recorded once they stabilized (less than one minute). The DO was also measured in uninoculated bottles containing media with and without reducing agents to measure the amount of abiotic oxygen reduction; uninoculated medium was exposed to either 0 or 8% $O_2$. Total carbon dioxide in the media was measured using a Bioprofile 400 Analyzer (Nova Biomedical, Waltham, MA, USA) per the manufacturer's instructions. Total $CO_2$ was calculated using the equation $tCO2 = [HCO_3^-] + \alpha * pCO_2$, where the solubility coefficient of $CO_2$ ($\alpha$) is estimated to be 0.0307.

**mRNA isolation, cDNA library preparation, and sequencing**

*C. ljungdahlii* was grown as described above in mRCMf with a syngas headspace and exposed to 0 or 8% $O_2$. Three culture bottles per experimental condition (0 and 8% $O_2$ exposed) were transferred into the anaerobic chamber at 14 h (2 h after oxygen exposure, early response) and 36 h (12 h after oxygen exposure, late response) time points (twelve samples total). Serum bottles were opened and 0.5 ml of culture was added to 1 ml of Qiagen RNA Protect Bacterial Reagent (Qiagen, Venlo, Limburg, Netherlands). These samples were centrifuged (10 min, 21,460 x g, 4°C); the supernatant was removed, and pellets were stored at -65°C. Batch cultures

used for RNA sequencing were not sampled for fermentation analysis to save as much culture for total RNA isolation as possible. To ensure that the liquid fermentation products for the cultures used for RNA sequencing were similar to the other fermentations in this study, liquid analysis (as described above) was performed on other cultures that were simultaneously inoculated.

The Qiagen RNeasy Mini Kit (Qiagen, Venlo, Limburg, Netherlands) with on-column RNAse-free DNase (Qiagen, Venlo, Limburg, Netherlands) treatment was used in conjunction with the enzymatic lysis and Proteinase K treatment (New England Biolabs, Ipswich, Massachusetts, USA) per the supplier's protocol to isolate total RNA. RNA quality was checked by gel electrophoresis. The Epicentre ScriptSeq Complete Kit (Bacteria) (Illumina, San Diego, California, USA) was used to isolate the mRNA as well as synthesize and amplify the cDNA with a different index for each sample (twelve total). The Qiagen RNeasy MinElute Kit (Qiagen, Venlo, Limburg, Netherlands) was used to purify the mRNA. mRNA and cDNA were analyzed for quality and concentration using an Agilent 2500 Bioanalyzer on an Agilent RNA 6000 Pico Chip and DNA High Sensitivity Chip, respectively, at the North Carolina State Genomic Sciences Lab (Raleigh, NC). Indexed cDNA was pooled and subjected to bioanalysis prior to sequencing at the BGI and Children's Hospital of Philadelphia collaborative genome facility using the 100bp Single Read protocol for one lane on the Illumina HiSeq2000. Base quality calls and demultiplexing were performed with the CASAVA 1.8.2 pipeline (Illumina, San Diego, California, USA).

**Differential expression analysis**

Differential analysis was performed with DEGseq as described in Tan *et al.* with exception of raw reads being counted with eXpress prior to importing into DEGseq (82, 98). Unless otherwise stated, genes mentioned in this paper were significantly differentially expressed



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based on a FDR (false discovery rate (7)) ≤ 0.001 and a normalized log2 fold change ≥ 2 or ≤ -2. Tablet software was used to visualize differentially regulated gene clusters (64, 65).

**Nucleotide sequence accession numbers**

RNA sequencing data for each condition were submitted to the NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra) under accession no. PRJNA296707.

**Nucleotide sequence alignment and database search**

Re-annotation of differentially expressed genes was performed using the blastx program (National Centre of Biotechnology Information [http://www.ncbi.nlm.nih.gov/BLAST]) using the default parameters. Multiple sequence alignments were performed with Vector NTI using the standard parameters (61).

**Enzyme assays**

As with cultures for RNAseq analysis, *C. ljungdahlii* cultures for enzyme assays were either treated with 0 or 8% $O_2$ at 12 h and samples for assays were taken at 14 h and 36 h for early and late response to oxygen exposure. At these time points, cultures were transferred to an anaerobic chamber and dispensed into centrifuge bottles (six 50 ml cultures per bottle). The bottles were sealed and centrifuged (25 min at 11,000 x *g*, 4 °C). The cell pellets were washed with 40 ml anaerobic potassium phosphate buffer (50 mM, pH 7.0) and centrifuged as described above. Cell pellets were resuspended in 3 ml anaerobic potassium phosphate buffer (50 mM, pH 7.0) per gram of wet cell pellet. Five hundred microliters of the cell suspension were transferred to 2 ml screw cap tubes. The cell suspensions were either processed immediately for assays or stored at -65°C.

For cell suspension processing, 500 µl of 0.1 mm disruption glass beads (RPI, Mt. Prospect, Illinois, USA) were added to the tubes. Samples were vortexed horizontally (10 min,

maximum speed) using a MoBio vortex adapter 13000-V1 for Vortex-Genie 2® (MoBio, Carlsbad, California, USA), and centrifuged (10 min, 21,000 x *g*, 4 °C). Supernatants were collected and kept on ice for assays. All assays were carried out in a 2 ml total volume (once all reagents were added) at 25 °C in anaerobic cuvettes except peroxidase and oxidase, which had a total volume of 3 ml, and the superoxide dismutase and xanthine oxidase assays, which were performed aerobically in 96-well plates. All enzyme assays are reported as an average of six replicates.

NAD(P)H oxidase was assayed as described by Stanton and Jensen (87). For a negative control, the assay was performed in anaerobic cuvettes with anoxic buffer and headspace. Reactions were initiated by adding cell extract (10-100 µg protein). Catalase was assayed by measuring the disappearance of $H_2O_2$ at 240 nm (27). The catalase reactions were initiated by adding cell extract (10-100 µg protein). Peroxidase was assayed as described by Poole and Ellis except that 200 µM NADH or NADPH (final concentration) was used (75). SOD activity was determined using a commercially available Water Soluble Tetrazolium (WST) salt SOD assay kit (Dojindo Laboratories, Kumamoto, Japan) per the manufacturer's instructions (72). Superoxide dismutase from *Bovine erythrocytes* (MP Biomedicals, Santa Ana, CA, USA) was used as a positive control. Xanthine oxidase activity was assayed with the Amplex Red Xanthine/Xanthine Oxidase Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) per the manufacturer's instructions. Xanthine was omitted from the reaction mixture as a negative control.

Alcohol dehydrogenase assays contained 1.5 ml 100 mM Tris-HCl (pH 8.5), 0.5 ml 2M ethanol, and 1 ml of 0.025 M $NAD^+$ or $NADP^+$. Reactions were initiated by adding cell extract (10-100 µg protein), and the appearance of NADH or NADPH was measured at a wavelength of 340 nm. Acetaldehyde dehydrogenase assays were performed as described in (14). The reverse



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reaction of aldehyde oxidoreductase (AOR), carboxylic acid reduction (CAR), was performed as described in (28) to measure acetate reduction, except that the assay was performed at 25 °C in 500 mM potassium phosphate (pH 6.0) and the electron donor was methyl viologen (140 μM), which was completely reduced by dithionite (150 μM). The forward reaction, acetaldehyde-dependent reduction of benzyl viologen (aka acetaldehyde oxidase), was also measured. Sodium dithionite was not used for the forward reaction. Unreduced cultures were also used as a control to avoid reduction of benzyl viologen by the reducing agents rather than by the cell-free extracts.

For all assays, activities (units/mg) are defined as μmoles of substrate (e.g. methyl violgen, benzyl viologen, $NAD(P)^+$, NAD(P)H) oxidized or reduced per minute. Methyl viologen oxidation and benzyl viologen reduction were quantified by measuring their absorbance at 600 nm and using a molar extinction coefficient of 12,000 $M^{-1}$ $cm^{-1}$ or 7,400 $M^{-1}$ $cm^{-1}$, respectively. NAD(P)H oxidation and $NAD(P)^+$ reduction were quantified by measuring their absorbance at 340 nm and using a molar extinction coefficient of 6,220 $M^{-1}$ $cm^{-1}$.

**Electrophoresis and protein identification by MALDI-TOF/TOF and Mascot analysis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% acrylamide gels by the procedure developed by Laemmli (55). 14 and 36 h cell-free cell extract samples from 0 and 8% $O_2$ exposed cultures, including cultures grown with reduced and unreduced medium, were boiled for 10 minutes with loading dye and run on SDS-polyacrylamide gels for 45 minutes at 200 V. Protein bands were detected by staining the gel with Coomassie brilliant blue G-250. The gel was submitted to the UNC Michael Hooker Proteomics Center (University of North Carolina, Chapel Hill, NC, USA); the band of interest was excised, trypsin-digested, and analyzed by MALDI-TOF/TOF. MS/MS spectra were



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searched for proteins against the NCBI database for *C. ljungdahlii* DSM13528 (Accession CP001666) using Mascot software (Matrix Science Inc, Boston, MA 02110).

**Growth and Cell Protein Quantification**

Growth was measured as optical density at 600 nm. Protein quantification in cell extracts was performed using Bio-Rad's Protein Assay Dye Reagent (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

To determine the relationship between optical density and dry cell density, 11 ml of culture was sampled from growing cells at various time points. One ml was used for $OD_{600}$ readings while 10 ml of culture was filtered through a disposable pre-weighed and pre-dried 0.22 µm syringe filter. Flow through was discarded. Filter and cells were washed with 10 ml 50 mM phosphate buffer pH 7.0. The filters were then incubated at 80°C in a dry air oven and were weighed every 24 h until constant weights were obtained. The optical densities were plotted against their corresponding dry cell weights yielding a linear relationship between measured $OD_{600}$ nm and culture densities (mg dry cells/L). The relationship between optical density and dry cell weight was found to be 312 mg dry cells/L per $OD_{600}$ unit for *C. ljungdahlii*.

## RESULTS

**Effects of oxygen on growth and fermentation product formation**

*C. ljungdahlii* was previously shown to be consistently resistant to concentrations of oxygen as high as 8% (volume added/volume headspace) injected into the headspace of batch cultures with mixotrophic medium (94). This finding was confirmed in this study (Figure 1A). Though cultures exposed to 8% $O_2$ continued to grow, the rate of growth was reduced compared to anaerobic cultures (generation time of 5.8 h vs. 4.6 h) (Figure 1A). Similar to previous

findings, more ethanol and less acetate were produced by cultures exposed to 8% $O_2$ as well (1:2 vs. 1:6 mol-ethanol:mol-acetate) (Figure 1B and 1C) (94). Substrate utilization of CO, $H_2$, and fructose continued in 8% $O_2$ exposed cultures but the rate was slower than in anaerobic cultures, and 8% $O_2$ exposed cultures consumed about 13% less hydrogen than anaerobic cultures (Figure 2A). By 36 h (24 h after oxygen exposure), CO, $H_2$, and fructose consumed per liter of culture for oxygen exposed cells were 7%, 19%, and 25% lower than anaerobic cultures, respectively.

$CO_2$ in both 8% $O_2$ exposed and anaerobic cultures increased above initial concentrations by 14 h and continued to increase throughout the fermentation to 72 h (Figure 2B). However, there was a substantial difference in the amount of $CO_2$ per liter of culture in 8% $O_2$ exposed batch cultures and anaerobic batch cultures, 0.577 g by 72 h (Figure 2B).

Oxygen injected into the headspace was confirmed to be removed from the inoculated cultures completely by 36 h (Figure 2C). The total amount of oxygen added to cultures and reduced by the cells was calculated to be ~ 6.9 mmol/L of culture (data not shown). However, based on dissolved oxygen (DO) concentrations of unreduced uninoculated and (reduced) uninoculated medium controls, a portion of this was reduced by cysteine-HCl and sodium sulfide (reducing agents) added to the medium (Figure 2C). Reducing agents kept the amount of DO in the uninoculated medium close to 0 ppm for at least 2 h after oxygen was injected into the headspace (Figure 2C). Sometime between 14 and 30 h, the reducing agents were used up, and the concentration of DO in the uninoculated medium increased to ~2.4 ppm (Figure 2C). The DO in the unreduced uninoculated medium also increased between the 14 and 30 h time points indicating that it took longer than 2 h for the DO in the liquid and gas phases to reach equilibrium (Figure 2C). In this study, reducing agents were added to the medium because ethanol production was higher than when it was excluded, but reducing agents are not required



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for continued growth of *C. ljungdahlii* in rich mixotrophic medium when exposed to 8% $O_2$ (94). Based on a comparison of DO in uninoculated medium containing reducing agents and uninoculated medium without reducing agents, reducing agents removed at most 0.9 ppm (39%) of the oxygen by 36 h (Figure 2C). This means at least 4.2 mmol/L of oxygen was removed by the *C. ljungdahlii* cells (data not shown). At the same time, ~13.1 mmol/L (0.577 g/L) less $CO_2$ was reduced by 8% $O_2$ exposed cells.

**Effects of oxygen on gene expression**

RNA sequencing was employed in an effort to identify genes responsible for the oxygen tolerance and higher ethanol/acetate ratio of batch cultures exposed to 8% $O_2$. Samples for sequencing were taken at 14 h (2 hours after oxygen exposure, early response) and 36 h (24 h after oxygen exposure, late response) for 8% $O_2$ exposed and anaerobic cultures. In total, 139,256,223 reads with an average length of 100 bp were generated (Table S1) with each treatment condition representing greater than 28.9 million reads. Differential expression analysis was conducted using DEGseq as in a previous RNAseq study with *C. ljungdahlii* (90). All 150 differentially expressed genes are displayed in Table S2.

The expression profile of *C. ljungdahlii* genes known and predicted to be involved in ethanol and acetate production are presented in Table 1. Though AdhE1 (encoded by CLJU_c16510) was previously identified to be the primary ethanol dehydrogenase in *C. ljungdahlii* (4, 57), its gene was not differentially expressed at 14 or 36 h; neither were the genes for phosphotransacetylase (CLJU_c12770) or the acetate kinase (CLJU_c12780). Genes for predicted acetaldehyde dehydrogenases (CLJU_c11960, CLJU_c39730, CLJU_c39840), which may be involved in production or consumption of acetaldehyde, the precursor of ethanol, were also not differentially expressed. However, a predicted tungsten-containing aldehyde ferredoxin

oxidoreductase (CLJU_c20210), one of two previously hypothesized to catalyze acetate reduction to acetaldehyde with reduced ferredoxin (51), was expressed significantly less by 8% $O_2$ exposed cells than by anaerobic cells at 14 h. The other predicted tungsten-containing aldehyde ferredoxin oxidoreductase (CLJU_c20110) was not differentially expressed at 14 or 36 h. The only differentially expressed genes that showed potential for explaining higher ethanol production were a predicted aldehyde oxidoreductase (CLJU_c24130) with homology to molybdenum-containing AORs and a putative xanthine dehydrogenase (CLJU_c23910) with 50% identity to CLJU_c24130 (Table S3). Both were expressed significantly higher in 8% $O_2$ exposed cells than anaerobic cells at 36 h; however, expression of CLJU_c24130 was also higher (though not significantly) while expression of CLJU_c23910 was lower (though not significantly) in 8% $O_2$ exposed cells at 14 h, and the transcript count of CLJU_c24130 was about 9 times higher than CLJU_c23910 (Table 1). A predicted aldehyde oxidoreductase (CLJU_c24050) with 80% identity to CLJU_c24130 was not differentially expressed at 14 or 36 h (Table 1 and Table S3).

The genes that are predicted to be specifically involved in oxygen or reactive oxygen species (ROS) detoxification are provided in Table 2 and include two predicted rubrerythrins (CLJU_c28910) and (CLJU_c39340). Rubrerythrin is a common name used for NAD(P)H hydrogen peroxidases found in Clostridia and other anaerobic bacteria. The predicted rubrerythrin encoded by CLJU_c39340 has 80% identity with *C. acetobutylicum*'s highly active NAD(P)H hydrogen peroxidase, a "reverse rubrerythrin" also called "rubperoxin" (encoded by homologs CA_C3597 and CA_C3598) (Table S4) (33, 63, 81). *C. acetobutylicum*'s rubrerythrin is indirectly reduced by NAD(P)H (preferentially by NADH) through a NADH:rubredoxin oxidoreductase (NROR) and a rubredoxin (41). An alignment of all the genes encoding



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differentially expressed rubrerythrin(-like) enzymes from *C. ljungdahlii*, CLJU_c09090 (differentially expressed gene of the FAD/NAD-dependent oxidoreductase subunit of the anaerobic-type CODH), and *C. acetobutylicum* NROR (CA_C2448) and rubredoxin (CA_C2778) showed less than 40% identity for all combinations (Table S4).

A putative flavoprotein (CLJU_c21940) and a hemerythrin related protein (CLJU_c36090) are also listed in Table 2. The CLJU_c21940 flavoprotein has 60 % identity to a *C. acetobutylicum* flavoprotein (CA_C2449) that has demonstrated oxidase activity and is reduced by NROR (Table S4) (41). Hemerythrins are proteins that carry oxygen and/or convert oxygen to hydrogen peroxide (38, 103). *C. ljungdahlii* also has an annotated superoxide dismutase (CLJU_c29780), but expression of this gene was low for both 8% $O_2$ exposed and anaerobically cultured cells (data not shown).

Besides genes directly involved in ethanol/acetate production and oxygen/ROS detoxification, several genes related to substrate, energy, and cofactor metabolism were identified as being differentially expressed (Table 3). These include genes encoding the anaerobic-type CODH complex (CLJU_c09090-110) which are thought to be responsible for CO oxidation to $CO_2$ in *C. ljungdahlii* (68), the G-subunit of the RNF complex (CLJU_c11380) which is predicted be a FMN binding redox active subunit responsible for reduction of NAD (51, 88), a glyceraldehyde 3-phosphate dehydrogenase (CLJU_c13400) which is a glycolysis enzyme, a hydrogenase expression/formation protein (CLJU_c23080) predicted to be involved in nickel insertion (51), the CODH of the Wood-Ljungdahl Pathway gene cluster (CLJU_c37670) which is predicted to bind CO for acetyl-CoA synthesis (51, 52, 68), a predicted citrate:cation symporter and a NAD-dependent malic enzyme potentially involved in the branched TCA "cycle" in *C. ljungdahlii* (51). Of these genes, the anaerobic CODH complex was significantly

more highly expressed by 8% $O_2$ exposed cells at both 14 and 36 h; the glyceraldehyde 3-phosphate dehydrogenase, the predicted citrate:cation symporter and NAD-dependent malic enzyme were significantly more highly expressed by anaerobic cells at 14 h; and the G-subunit of the RNF complex, the predicted hydrogenase expression/formation protein, and the CODH were significantly more highly expressed by 8% $O_2$ exposed cells at 36 h (Table 3). Several other genes related to peptide uptake and amino acid metabolism were also more highly expressed in 8% $O_2$ exposed cells at 14 h (CLJU_c19310-20, CLJU_c37340-50) and 36 h (CLJU_c28700, CLJU_c28730-60) (Table S2).

Table 3 also includes a Fur family transcriptional regulator (CLJU_c35550) expected to be involved in iron metabolism and a ten member gene cluster (CLJU_c19400-90) predicted to be involved in metal cofactor repair and management (named *crm*). A KEGG image and visual representation of the differentially expressed *crm* gene cluster is provided in Figure 3A and 3B, respectively. The *crm* gene cluster includes a predicted transcriptional regulator (CLJU_c19400), a ferric uptake regulation protein (CLJU_c19410), a predicted two-component response regulator (CLJU_c19450) and a predicted signal transduction histidine kinase (CLJU_c19460). In addition to these, the hypothetical protein (CLJU_c19470) contains a polo-box domain of a polo-like kinase. Polo-like kinases have been known to be involved in cell cycle regulation, DNA damage and oxygen stress in other organisms (107).

Besides these regulatory proteins the gene cluster also contains a gene encoding ferritin (CLJU_c19490), a protein known to sequester iron, and a gene encoding a flavin reductase-like protein with a rubredoxin domain similar to proteins shown to release iron from ferritin using reduced FMN (CLJU_c19480) (8, 17, 36, 101). Flavins used by this protein may be transported into the cell by the putative membrane protein encoded by CLJU_c19430, which contains two



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EamA-domains like the characterized riboflavin transporting transmembrane permeases (RibN) from *Ochrobactrum anthropic* and *Vibrio cholera* (26). A blastx query of the putative epimerase gene (CLJU_c19420) in this oxygen-induced gene cluster revealed that it had higher homology to a phenazine biosynthesis-like protein than its annotated function as an epimerase. Phenazines serve as electron shuttles for various terminal electron acceptors and have been shown to reduce iron [hydr]oxides in *Pseudomonas chlororaphis* and other bacteria (29). Also, a blastx query of the gene sequence for the conserved hypothetical protein (CLJU_c19440) revealed a DUF1848 domain. The C-terminus of DUF1848 domains contain a cluster of cysteines similar to the Fe-S cluster of a radical SAM domain, which are known to catalyze diverse reactions including cofactor biosynthesis and maturation, post-transcriptional and post-translational modification, enzyme activation, substrate anchoring, electron transfer, and sulfur donation (56).

**Enzyme activities associated with ethanol production**

Figure 4A shows a modified version of the predicted *C. ljungdahlii* anabolic pathways leading to ethanol formation previously described by Köpke et al. (51). Either ethanol can be produced from acetyl-CoA by acetaldehyde dehydrogenase activity followed by ethanol dehydrogenase activity or from acetate by acetate reductase (reverse reaction of aldehyde oxidation) followed by ethanol dehydrogenase (Figure 4A).

Acetate reductase activity was at least 6-fold higher at 14 h and 4-fold higher at 36 h on average for cell-free cell extracts from 8% $O_2$ exposed cultures compared to cell-free extracts from anaerobic cultures (Figure 4B). Acetaldehyde oxidoreductase activity as measured by benzyl viologen reduction did not seem to be present in anaerobic or 8% $O_2$ cells at 14 or 36 h since acetaldehyde did not increase benzyl viologen reduction by cell-free extracts (data not shown). However, acetaldehyde-independent benzyl viologen reduction was mediated by cell-

free extracts of 14 h 8% $O_2$ exposed cultures, 36 h anaerobic cultures, and 36 h 8% $O_2$ exposed cultures, but not 14 h anaerobic cultures (Figure S2A). Aldehyde-independent benzyl viologen reduction by cell-free extracts indicates that cell-free extracts (except those derived from 14 h anaerobic cultures) have a lower redox potential than benzyl viologen. Also, based on a general trend of higher acetaldehyde-independent benzyl viologen activity for cell-free extracts from cells grown normally in media with reducing agents versus unreduced cultures, electrons from reducing agents can be used by cells to reduce benzyl viologen (Figure S2A). Ethanol and acetaldehyde dehydrogenase activities of cell-free extracts from 8% $O_2$ exposed and anaerobic cultures had less than 2-fold difference at 14 and 36 h time points (Figure S2B and S2C).

**Enzyme activities associated with oxygen tolerance**

Based on differential expression of genes for multiple rubrerythrin and rubrerythrin-like enzymes (hemerythrin) (Table 2), it was hypothesized that the 8% $O_2$ exposed *C. ljungdahlii* cultures would have higher NAD(P)H hydrogen peroxidase activity than anaerobic cultures. Figure 5A shows that this prediction was correct at both 14 and 36 h.

*C. ljungdahlii* was also assayed for catalase, oxidase, superoxide dismutase, and xanthine oxidase activities which are other common enzyme activities for ROS and oxygen detoxification. No detectable activity was observed for these enzymes in cell-free extracts of 8% $O_2$ exposed or anaerobically-grown *C. ljungdahlii* cells (data not shown).

**Identification of an oxygen inducible protein**

To determine if there were any observable highly translated oxygen-induced proteins, cell-free extracts from anaerobic and 8% $O_2$ exposed cultures (14 and 36 h) were run on a SDS-PAGE gel (Figure 5B). A distinct band between the ~15 and ~25 kD ladder markers was present only in lanes 3 and 4 corresponding to the 14 h and 36 h 8% $O_2$ exposed cultures (Figure 5B).

Cell-free extracts from anaerobic and 8% $O_2$ exposed cultures (14 and 36 h) from cells grown in media without reducing agents were also run on a SDS-PAGE gel (Figure S1); no apparent difference was observed between these and cell-free extracts from cells grown in medium containing reducing agents. The distinct band was identified to be the differentially expressed predicted rubrerythrin (CLJU_c39340) (Figure 5C, Table 1).

## DISCUSSION

In this study, exposure of *C. ljungdahlii* to 8% $O_2$ when grown in mixotrophic medium was confirmed to result in higher ethanol production with corresponding lower acetate production (Figure 1B and 1C) (94). Differential expression analysis and enzyme activity assays revealed two genes (CLJU_c24130 and CLJU_c39340) that are likely to play major roles in higher ethanol production and oxygen detoxification, respectively (Tables 1 and 2; Figure 4B and 5A). Expression analysis and fermentation products also suggest that carbon, energy and cofactor metabolism link the AOR and rubrerythrin activities (Table 3).

Higher ethanol production is due to a carbon flux from reduction of acetate to acetaldehyde by AOR followed by reduction of acetaldehyde to ethanol by alcohol dehydrogenase (likely AdhE1) (Figure 4A). The primary enzyme found to be responsible for higher ethanol production in 8% $O_2$ exposed cultures compared to anaerobic cultures appears to be an AOR (encoded by CLJU_c24130). In addition to significantly higher expression of this gene in 8% $O_2$ exposed cells, it was also shown that AOR activity (acetate reductase activity) was 6 and 4 times higher in 8% $O_2$ exposed cells compared to anaerobic cells at 14 and 36 h, respectively (Table 1 and Figure 4B). However, a recent study by Xie *et al.* showed that higher expression of another AOR (encoded by CLJU_c20110) correlated with higher gene expression

and higher ethanol production by *C. ljungdahlii* (102). Prior to this study, *Kopke et al.* predicted that under surplus reducing equivalents, acetate could be converted to acetaldehyde, the precursor of ethanol, by the AOR's encoded by CLJU_c20110 and CLJU_c20210 (51). It was also shown that CLJU_c20110 was significantly more highly expressed when *C. ljungdahlii* was grown autotrophically (80% CO, 20% $CO_2$) than when it was grown heterotrophically with fructose (59, 102). In contrast, we observed that both CLJU_c20110 and CLJU_c20210 had relatively low transcript counts which may be explained by the growth medium used in this study having fructose as the primary carbon source (Table 1). In fact, our results indicate that CLJU_c20210 was significantly more highly expressed in anaerobic cultures than 8% $O_2$ exposed cultures at 14 h (Table 1). To our knowledge, this is the first study to demonstrate corroborating fermentation, expression and activity data associated with a particular AOR (encoded by CLJU_c24310) which is a different one than the other AOR's reported (Table 1, Figure 1B and 4B).

As with the AORs encoded by CLJU_c20110 and CLJU_c20210, the AOR encoded by CLJU_c24130 is expected to utilize ferredoxin as a coenzyme for activity (99). However, this AOR is predicted to use molybdenum as a cofactor, whereas the other two are predicted to be tungsten-containing AORs (51). Of the characterized carboxylic acid reducing AORs of *C. formicoaceticum*, a molybdenum-containing AOR is much less oxygen labile than the tungsten-containing AORs, and it is true in general that tungsten is more oxygen labile than molybdenum (44, 45, 76, 99, 109). More specifically, *C. formicoaceticum*'s purified tungsten-containing AOR lost 20% of its activity in 5 min versus 4% for the molybdenum-containing AOR (99). This may explain why the gene for the tungsten-containing AOR was significantly less expressed while the gene for the AOR encoded by CLJU_c24310 was significantly more highly expressed when

513 cells were exposed to 8% $O_2$ (Table 1). A drawback of molybdenum-containing AORs is that
514 they are less active than tungsten-containing AORs; in *C. formicoaceticum* the purified
515 molybdenum-containing AOR was found to have 10 times less activity than the tungsten-
516 containing AOR (99). While it is not a perfect comparison, here we found that even the higher
517 carboxylic acid reductase activity of cell-free extracts of 8% $O_2$ exposed cells (acetate reductase)
518 was about 4 times less than the (propionate reductase) activity of *C. ragsdalei* cell free extracts
519 (~0.03 U/mg vs ~0.12 U/mg) (Figure 4B). The cell-free extracts from anaerobic cultures were 24
520 times less than *C. ragsdalei* (~0.005 U/mg vs ~0.12 U/mg) (Figure 4B). The difference in
521 activities for anaerobic cultures of these closely related species can be explained by autotrophic
522 growth for *C. ragsdalei* versus mixotrophic growth with fructose as the predominant carbon
523 substrate for *C. ljungdahlii*. As previously mentioned, expression of *C. ljungdahlii*'s main
524 tungsten-containing AOR (CLJU_c20110) was significantly less for fructose-grown cells than
525 for 80% CO-grown cells (59, 102). Despite the lower activity of molybdenum-containing AORs,
526 their benefit of a higher tolerance to oxidized compounds like $O_2$, $NO_X$, and $SO_X$ may make this
527 predicted molybdenum-containing AOR a more interesting genetic target for overexpression in
528 commercial applications where less syngas purification means higher margins. Further study of
529 the substrate specificity of this molybdenum-containing AOR would also benefit those interested
530 in producing biochemicals with the carboxylate platform.

531 Another major finding of this study is the identification of a rubrerythrin (encoded by
532 CLJU_c39340) which is likely to play a key role in *C. ljungdahlii*'s ability to tolerate low
533 oxygen exposure by reducing hydrogen peroxide. Unlike several other genes encoding
534 rubrerythrin and rubrerythrin-like (hemerythrin) enzymes which were also differentially
535 expressed at 14 and 36 h, the protein encoded by CLJU_c39340 was also highly translated



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(Table 2 and Figure 5B). In a previous study, SDS-PAGE analysis of air-exposed *C. acetobutylicum* also showed one distinct band at 22 kDa, which was later revealed to be two rubrerythrins with one amino acid difference (encoded by CA_C3597 and CA_C3598) (40, 93). These rubrerythrins which were shown to be highly active hydrogen peroxidases have sequence and structural similarities to CLJU_c39340 (Table S4) (41). Actually, the hydrogen peroxidase activity was found to be about 40 times higher in *C. ljungdahlii* oxygen-exposed cell-free extracts than *C. acetobutylicum* oxygen-exposed cell free extracts (~0.93 U/mg vs ~0.023 U/mg) (Figure 5A) (42). However, it is unclear what [co]enzymes are acting as rubredoxin and NADH:rubredoxin oxidoreductase in *C. ljungdahlii* since no differentially expressed genes predicted to be involved in oxygen/ROS detoxification had higher than 40% homology with the respective [co]enzymes in *C. acetobutylicum* (Table S4). Despite the induction of protective oxygen-responsive genes and higher hydrogen peroxidase activity, *C. ljungdahlii* is much more sensitive to oxygen than *C. acetobutylicum* (Table 2, Figure 5A). Mid-log phase batch cultures of *C. acetobutylicum* (300 mg dry cells/L) were previously found to rapidly reduce 0.64-0.80 ppm DO in the growth media, and cells were able to survive after flushing with air for 30 min (~20% $O_2$) and even grow during flushing with 5% $O_2$ (40, 70). In contrast, *C. ljungdahlii* cultures required about 24 h to reduce 0.18 ppm DO and were previously shown to die after exposure to greater than 10% $O_2$ (Figure 2C) (94). The lower oxygen tolerance of *C. ljungdahlii* compared to *C. acetobutylicum* is likely the result of *C. ljungdahlii*'s lack of oxidase and superoxide dismutase/reductase activities which are present in *C. acetobutylicum* (data not shown) (33).

AOR and rubrerythrin activities (and thus the higher ethanol production and oxygen detoxification) are connected through carbon, energy, and cofactor metabolism. Both AOR and rubrerythrin are redox proteins that require the coenzymes reduced-ferredoxin or NAD(P)H for



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559 their respective activities. In *C. ljungdahlii*, ferredoxin can be reduced by CO oxidation activity,
560 hydrogenase activity, and PFOR (fructose metabolism) activity; NAD(P)+ can be reduced by
561 hydrogenase, transhydrogenase NfnAB (energy conservation), and RNF complex activity
562 (energy conservation) (84). Of these, expression analysis revealed that (at 14 and 36 h) the
563 anaerobic-type CODH and (at 36 h) the redox active G-subunit of the RNF complex were
564 significantly more highly expressed in 8% $O_2$ exposed cells compared to anaerobic cells (Table
565 3). Furthermore, CO consumption per gram of cells was also higher while hydrogen and fructose
566 consumption were lower in 8% $O_2$ exposed cells (Figure 2A). Therefore, it appears that CO is
567 preferentially used as a source of electrons for oxygen detoxification. The ability of 14 h cell-free
568 extracts from 8% $O_2$ exposed cultures to reduce benzyl viologen (without the addition of any
569 other substrate, i.e. acetaldehyde) and the inability 14 h cell-free extracts from anaerobic cultures
570 to do the same indicates that cells exposed to 8% $O_2$ generate surplus reduced coenzymes with
571 higher redox potentials than benzyl viologen such as reduced ferredoxin (Figure S2A).
572 Expression analysis indicates that ferredoxin is reduced by the anaerobic-type CODH (Table 3).
573 This is followed by NAD+ reduction by ferredoxin:NAD+ oxidoreductase (Rnf) activity
574 providing NADH for hydrogen peroxidase activity by rubrerythrin (Table 2, Table 3, Figure 4A).
575 Turn-over of surplus reduced coenzymes is achieved by ferredoxin:acetate reductase activity by
576 AOR followed by NADH:acetaldehyde dehydrogenase activity by AdhE1 (Table 1, Figure 4A,
577 Figure S2C). Therefore, despite less total liquid products because some electrons were used to
578 reduce $O_2$ instead of $CO_2$, these activities resulted in a higher ethanol to acetate ratio in 8% $O_2$
579 exposed cultures (Figure 1B, 1C, 2B, 2C).

580 Of these redox [co]enzymes mentioned, ferredoxin, the anaerobic-type CODH,
581 rubrerythrin, and AOR require Fe-S clusters. When *C. ljungdahlii* is exposed to oxygen, the *crm*



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582 gene cluster is significantly upregulated (Table 3 and Figure 3). Based on blastx query results, it
583 is predicted to have a similar function as the *suf* operon in *E. coli*, which has been shown to be
584 involved in the regeneration or replacement of cofactors such as iron-sulfur clusters and
585 molybdopterin of molybdenum and tungsten-containing proteins, and limits Fenton reactions
586 during oxygen detoxification (71). Also, several other genes annotated as molybdopterin
587 biosynthesis and molybdopterin-containing proteins were significantly more highly expressed in
588 8% $O_2$ exposed cells (CLJU_c17950, CLJU_c20050-60, CLJU_c23910, CLJU_c24130) (Table
589 S2). Molybdopterin may contain either tungsten or molybdenum metals, but as previously
590 mentioned, molybdenum is less oxygen labile (44, 45, 76, 83, 109). Nickel may also be a more
591 preferential cofactor during oxygen exposure based on significantly higher expression of the
592 anaerobic-type (nickel-dependent) CODH complex (CLJU_c09090-110) at 14 and 36 h and
593 predicted hydrogenase expression/formation protein (CLJU_c23080) at 36 h, which is thought to
594 be involved in nickel insertion (Table 3) (51). In general, nickel-containing CODHs and
595 hydrogenases are oxygen labile, but some have long inactivation half-lives, can be protected by
596 the presence of substrate, and can be regenerated by reducing agents (13, 53, 85). The shift in
597 cofactors and associated enzymes allows for continued activity and less oxidative damage during
598 oxygen detoxification.

599 The ability of *C. ljungdahlii* to tolerate low concentrations of oxygen and other
600 oxidized contaminants (i.e. $NO_x$ and $SO_x$) is important for its application in the biofuels industry
601 because it is likely that such contaminants will be contained in biomass-derived syngas (18, 21,
602 104, 108). Although it was observed that exposure of *C. ljungdahlii* to low levels of oxygen (8%
603 $O_2$) increased ethanol and decreased acetate concentrations, the purpose of this work was not to
604 suggest that oxygen should be added to syngas fermentations. Slower metabolism of syngas



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605 components $H_2$ and CO and less carbon from CO going to product formation (through acetyl-
606 CoA synthesis) is undesirable; therefore, oxygen should generally be removed from *C.*
607 *ljungdahlii* syngas fermentations. Rather, this work contributes the identification of genetic
608 targets to potentially improve ethanol production in the presence of syngas contaminants by
609 improving the robustness of syngas-consuming microbial catalysts like *C. ljungdahlii* and thus
610 reduce the need for costly syngas-cleaning. This work also contributes new knowledge about the
611 metabolism and physiology of this model organism.
612

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622

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## FIGURE LEGENDS

**Figure 1.** *C. ljungdahlii* cell growth (A), ethanol (B) and acetate production (C) when cultures are exposed to 0 (circle) and 8% $O_2$ (square). 160 ml batch reactors have a 110 ml headspace volume. 8% $O_2$ was added to the headspace at 12 h (arrow). The data represent an average of three biological replicates per condition. Error bars indicate standard deviation.

**Figure 2.** *C. ljungdahlii* CO (square), $H_2$ (circle), and fructose (triangle) utilization in response to 0% $O_2$ (anaerobically grown, closed symbols) and 8% $O_2$ (open symbols) supplementation of the headspace gas. The data represent an average of six biological replicates per condition (A). Total $CO_2$ concentration (g/L) in 14 h 0% $O_2$ exposed, 14 h 8% $O_2$ exposed, 36 h 0% $O_2$ exposed, and 36 h 8% $O_2$ exposed *C. ljungdahlii* batch cultures. $tCO_2 = pCO_2headspace + [HCO_3^-] + \alpha * pCO_2medium$, where α (estimated solubility coefficient) = 1.3511 mg/L/mmHg. Uninoculated bottles were used as controls. The data represent an average of three biological replicates per condition (B). Concentration of dissolved oxygen (ppm) at the bottom of 8% $O_2$ exposed *C. ljungdahlii* batch cultures. 0% $O_2$ exposed cultures were used as blanks, and uninoculated bottles were used as controls. Reducing agents (Cys-HCl and $Na_2S$) were omitted

in unreduced medium. The data represent an average of three biological replicates per condition (C). 8% $O_2$ was added to the headspace at 12 h (arrow). 160 ml batch reactors have a 110 ml headspace volume. Error bars indicate standard deviation.

**Figure 3.** KEGG image of the *C. ljungdahlii* cofactor repair and management (*crm*) gene cluster (A). Visual comparison of read alignment to a locus on the *C. ljungdahlii* genome containing the *crm* gene cluster of FastQ reads derived from RNA sequencing of 0% exposed (anaerobically grown) and 8% $O_2$ exposed cultures (B). Images were generated using sequence assembly visualization software, Tablet. Sample #1 is one of three 14 h time point anaerobic cell transcriptomes, and sample #7 is one of three 14 h time point 8% $O_2$ exposed cell transcriptomes. Arrows 1, 2, 3, 4, and 5 point to positions 2107698 (beginning of the CLJU_c19400 ORF), 2111421 (end of the CLJU_c19440 ORF), 2111708 (beginning of the CLJU_c19450 ORF), 2115295 (end of the CLJU_c19470 ORF), and 2116834 on the genome (beginning of the CLJU_c19490 ORF).

**Figure 4.** Predicted *C. ljungdahlii* anabolic pathways leading to ethanol. Aldehyde oxidoreductase (AOR), acetate, acetaldehyde, Fdox/red (oxidized and reduced ferredoxin), Anaerobic-type CODH Complex, CO, $CO_2$, and ethanol are bold to indicate metabolic flux proposed to account for higher ethanol and lower acetate production by 8% $O_2$ exposed *C. ljungdahlii* batch cultures. Figure was modified from *Köpke et al.*, 2010. Pta (phosphotransacetylase), Ack (acetate kinase), ATP (adenosine triphosphate), AdhE (bifunctional aldehyde/alcohol dehydrogenase), 2 [H] (reduced form of nicotinamide adenine dinucleotide (phosphate)), PFOR (pyruvate:ferredoxin oxidoreductase), CODH (carbon monoxide dehydrogenase) (A). AOR (methyl viologen acetate oxidoreductase activity) enzyme activity assay results for cell-free cell extract from 14 h 0% $O_2$ exposed (anaerobically grown),

960 14 h 8% $O_2$ exposed, 36 h 0% $O_2$ exposed, and 36 h 8% $O_2$ exposed *C. ljungdahlii* batch
961 cultures. Activity (units/mg) is defined as µmoles of methyl violgen oxidized per minute (B).
962 The data represent an average of six biological replicates per condition. Error bars indicate
963 standard deviation.

964 **Figure 5.** NAD(P)H hydrogen peroxidase enzyme activity assay results for 14 h 0% $O_2$ exposed
965 (anaerobically grown), 14 h 8% $O_2$ exposed, 36 h 0% $O_2$ exposed, and 36 h 8% $O_2$ exposed
966 samples. Activity (units/mg) is defined as µmoles of NAD(P)H oxidized per minute. The data
967 represent an average of six biological replicates per condition. Error bars indicate standard
968 deviation (A).SDS-PAGE analysis of *C. ljungdahlii* cell-free cell extract (5 µg) from 14 h 0% $O_2$
969 exposed (Lane 2), 14 h 8% $O_2$ exposed (Lane 3), 36 h 0% $O_2$ exposed (Lane 4), and 36 h 8% $O_2$
970 exposed (Lane 5) cultures. 5 µl of PageRuler Prestained Protein Ladder were loaded into lanes 1
971 and 6. Image was modified to remove irrelevant lanes (B). Protein identification by MALDI-
972 TOF/TOF and Mascot analysis of $O_2$-induced band (denoted by an arrow in A). Significant score
973 identification threshold ($p<0.05$) for the *C. ljungdahlii* protein database is 72 (MS/MS and
974 peptide sequenced ion scores higher than this are considered a significant identification); the
975 MS/MS score and peptide sequenced ion score for CLJU_c39340 (locus tag for a predicted
976 rubrerythrin) are 726 and 697, respectively (C).
977



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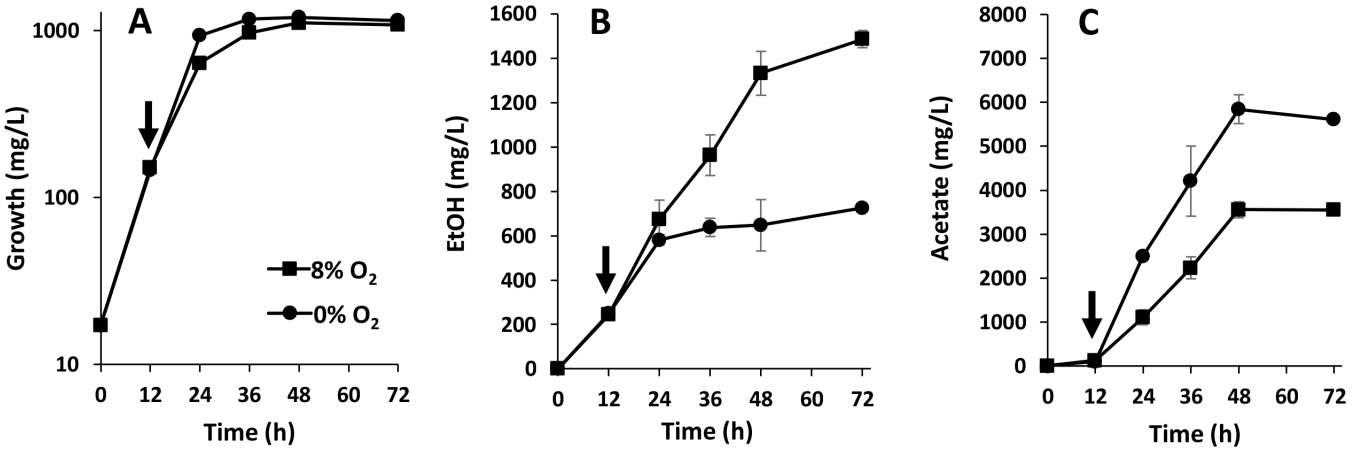

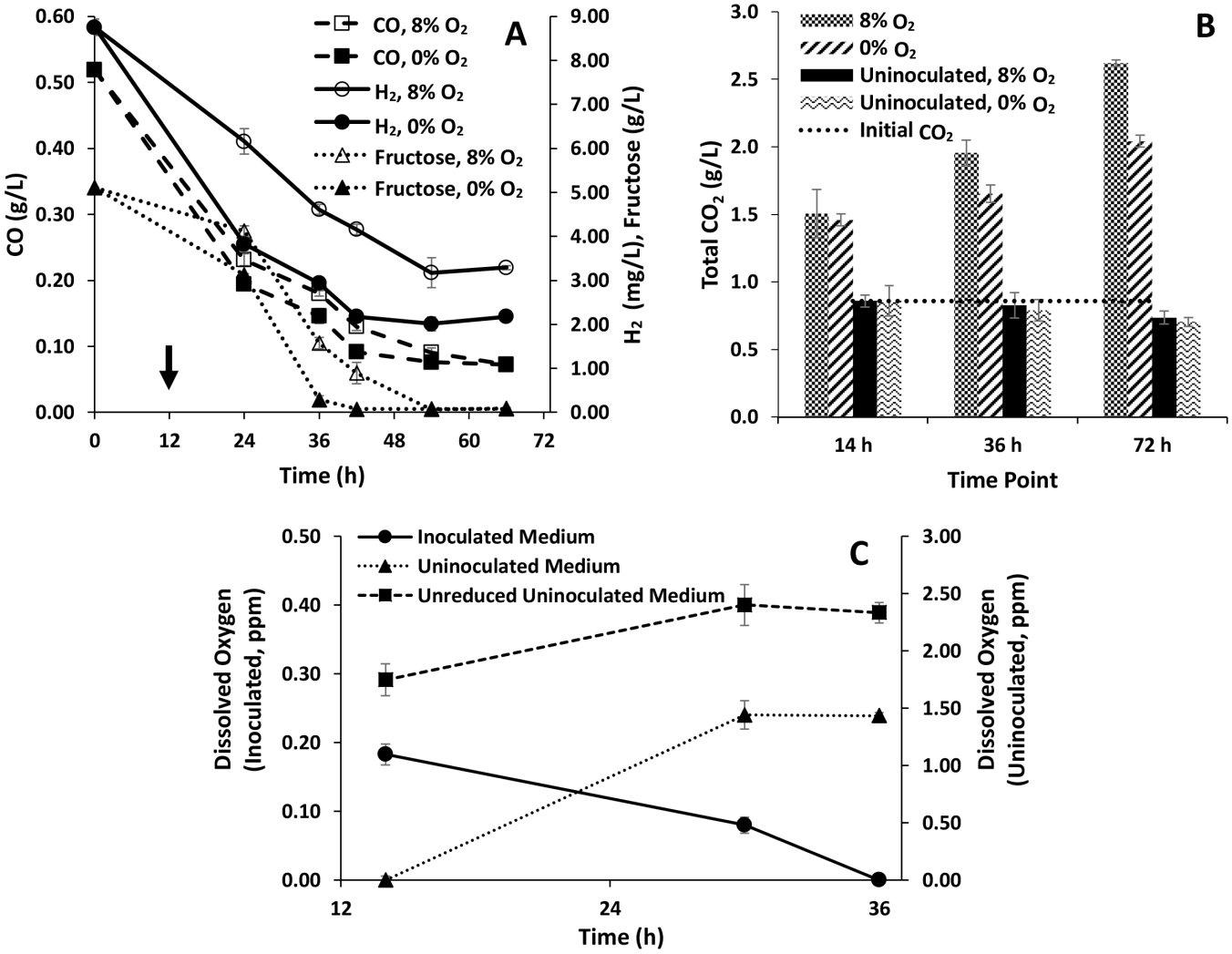

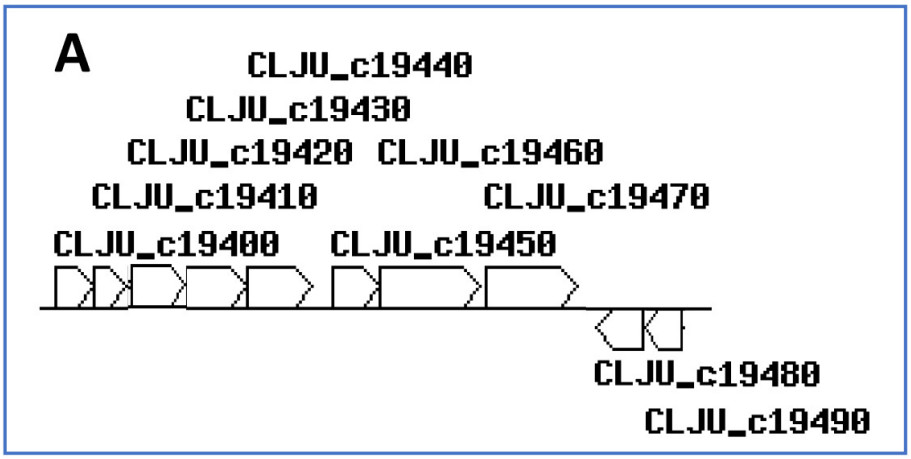


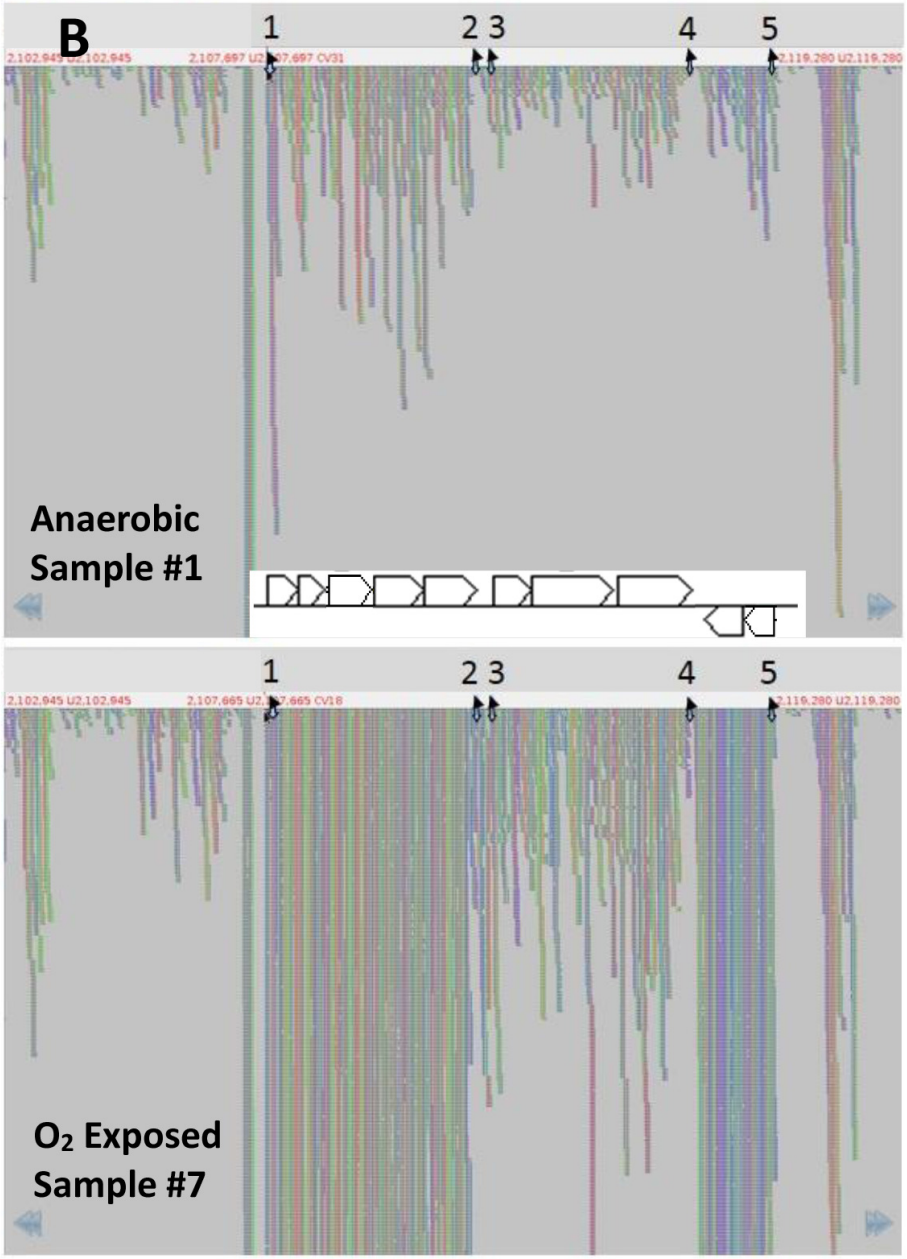

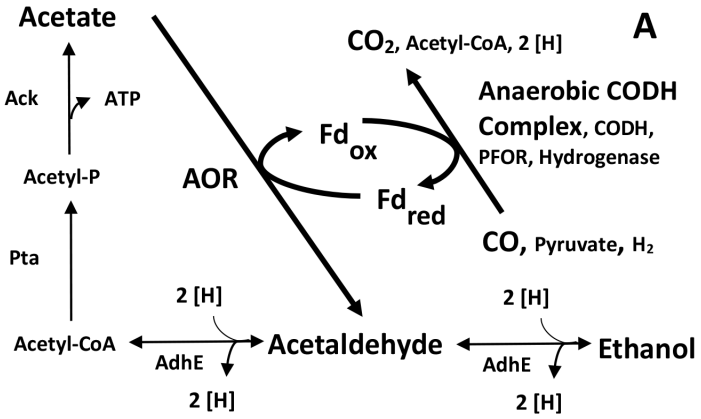


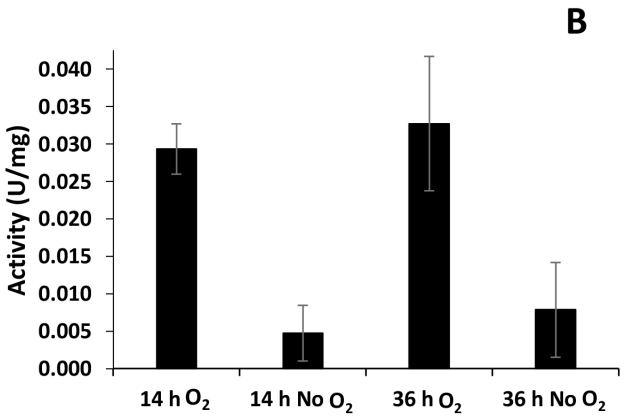

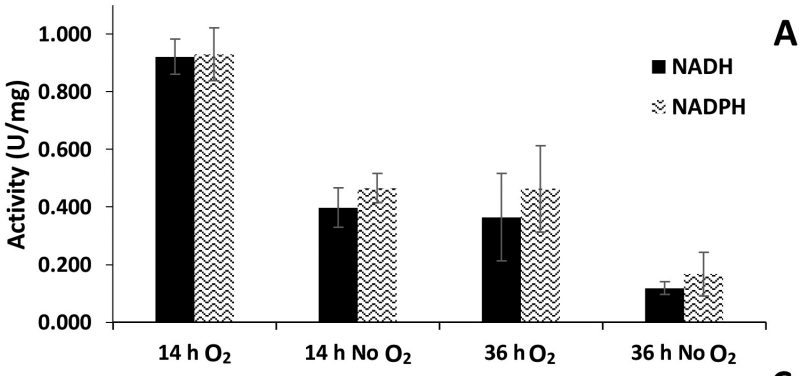


C

1 MKKFVCAVCG YVYEGENPPEKCPVCGAPSDKFVK**KAEGEI**
41 **SWADEHK**VGVAKGVDEK**VLEELR**ANFTGECSEVGMYLAMS
81 R**QADREGYPEVAEAYKRIAFEEAEHAAKFA ELLGEVVLPD**
121 **TKK**NLQMRVDAETGACKGKKDLATLAKKLNYDAIHDTVHE
161 MCKDEAR**HGAAFQGLLNR**YFK **(Rubrerythrin, CLJU_c39340)**

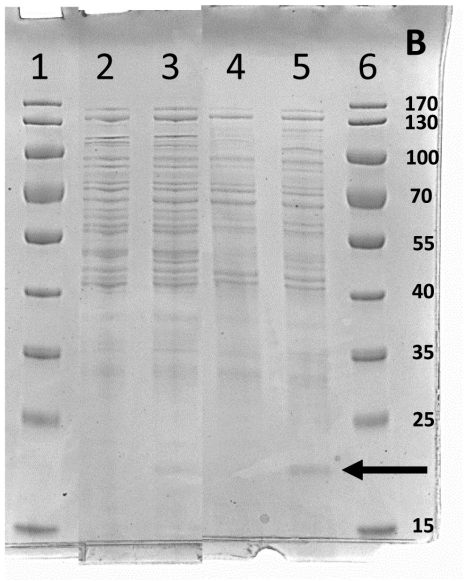

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**Table 1. Expression profile of C. *ljungdahlii* genes known and predicted to be involved in ethanol and acetate production.**

| Locus Tag | Gene Annotation | 14h 0% $O_2$ Count | 14h 8% $O_2$ Count | 14h Log2 F.C.† | 36h 0% $O_2$ Count | 36h 8% $O_2$ Count | 36h Log2 F.C. |
|---|---|---|---|---|---|---|---|
| CLJU_c11960 | predicted acetaldehyde dehydrogenase | 666 | 1148 | -0.355 | 32 | 60 | -0.530 |
| CLJU_c12770 | phosphotransacetylase | 37052 | 38122 | 0.388 | 51989 | 49972 | 0.468 |
| CLJU_c12780 | acetate kinase | 45467 | 43101 | 0.506 | 45696 | 48908 | 0.313 |
| CLJU_c16510 | bifunctional aldehyde/alcohol dehydrogenase (AdhE1) | 136600 | 184497 | -0.004 | 4954 | 2901 | 1.183 |
| CLJU_c20110 | predicted tungsten-containing aldehyde ferredoxin oxidoreductase | 64 | 52 | 0.708 | 549 | 645 | 0.181 |
| CLJU_c20210 | predicted tungsten-containing aldehyde ferredoxin oxidoreductase | 6547 | 1509 | 2.546 | 2055 | 1820 | 0.587 |
| CLJU_c23910 | putative xanthine dehydrogenase, molybdopterin-binding subunit B | 4519 | 3648 | 0.738 | 1798 | 11958 | -2.327 |
| CLJU_c24050 | predicted aldehyde oxidoreductase | 56 | 93 | -0.309 | 1058 | 896 | 0.650 |
| CLJU_c24130 | predicted aldehyde oxidoreductase | 9500 | 33544 | -1.391 | 9697 | 102536 | -2.991 |
| CLJU_c39730 | predicted acetaldehyde dehydrogenase | 6 | 6 | 0.635 | 2 | 3 | 0.070 |
| CLJU_c39840 | predicted acetaldehyde dehydrogenase | 601 | 784 | 0.045 | 686 | 623 | 0.550 |

† Comparisons in gene expression were made between anaerobic and 8% $O_2$ exposed cells. A positive N.Log2 F.C. (normalized Log2 fold change) value reflects higher expression by anaerobic cells and a negative N.Log2 F.C. value reflects higher expression 8% $O_2$ exposed cells. The threshold for comparison was set at an N.Log2 F.C. value of +/- 2.

**Table 2. Expression profile of genes differentially expressed by C. *ljungdahlii* predicted to be involved in oxygen and reactive oxygen species detoxification.**

| Locus Tag | Gene Annotation | 14h 0% $O_2$ Count | 14h 8% $O_2$ Count | 14h Log2 F.C.† | 36h 0% $O_2$ Count | 36h 8% $O_2$ Count | 36h Log2 F.C. |
|---|---|---|---|---|---|---|---|
| CLJU_c21940 | putative flavoprotein | 10478 | 204461 | -3.857 | 6207 | 36739 | -2.154 |
| CLJU_c28910 | predicted rubrerythrin | 1491 | 27416 | -3.771 | 885 | 16893 | -3.844 |
| CLJU_c36090 | hemerythrin related protein | 2353 | 59405 | -4.229 | 1263 | 9066 | -2.433 |
| CLJU_c39340 | predicted rubrerythrin | 19381 | 195485 | -2.905 | 39167 | 159979 | -1.619 |

† Comparisons in gene expression were made between anaerobic and 8% $O_2$ exposed cells. A positive N.Log2 F.C. (normalized Log2 fold change) value reflects higher expression by anaerobic cells and a negative N.Log2 F.C. value reflects higher expression 8% $O_2$ exposed cells. The threshold for comparison was set at an N.Log2 F.C. value of +/- 2.

**Table 3. Expression profile of genes differentially expressed by C. *ljungdahlii* known and predicted to be involved in substrate, energy, and cofactor metabolism.**

| Locus Tag | Gene Annotation | 14h 0% $O_2$ Count | 14h 8% $O_2$ Count | 14h Log2 F.C.† | 36h 0% $O_2$ Count | 36h 8% $O_2$ Count | 36h Log2 F.C. |
|---|---|---|---|---|---|---|---|
| CLJU_c09090 | anaerobic-type CODH, FAD/NAD-dependent oxidoreductase sub. | 15022 | 184044 | -3.186 | 7043 | 68954 | -2.880 |
| CLJU_c09100 | anaerobic-type CODH, e- transfer sub. | 4988 | 18436 | -1.457 | 2725 | 27886 | -2.944 |
| CLJU_c09110 | anaerobic-type CODH | 16453 | 274126 | -3.629 | 8432 | 92640 | -3.047 |
| CLJU_c11380 | RnfG | 12694 | 13522 | 0.338 | 670 | 10725 | -3.548 |
| CLJU_c13400 | glyceraldehyde 3-phosphate dehy. | 15196 | 3997 | 2.356 | 21082 | 49392 | -0.817 |
| CLJU_c17950 | putative molybdopterin biosyn. protein | 1625 | 2806 | -0.358 | 1944 | 21178 | -3.034 |
| CLJU_c19400 | predicted transcr. regulator | 2438 | 71339 | -4.442 | 5622 | 30966 | -2.050 |
| CLJU_c19410 | ferric uptake regulation protein | 1336 | 43688 | -4.602 | 4144 | 23877 | -2.115 |
| CLJU_c19420 | putative epimerase | 2040 | 38862 | -3.822 | 2688 | 17482 | -2.290 |
| CLJU_c19430 | putative membrane protein | 2187 | 38238 | -3.699 | 1918 | 14008 | -2.457 |
| CLJU_c19440 | conserved hypothetical protein | 3082 | 54143 | -3.706 | 3467 | 23993 | -2.380 |
| CLJU_c19450 | predicted two-comp. response reg. | 469 | 1549 | -1.294 | 316 | 1091 | -1.375 |
| CLJU_c19460 | predicted signal trans. histidine kinase | 1134 | 4242 | -1.474 | 800 | 2404 | -1.176 |
| CLJU_c19470 | hypothetical protein | 1415 | 4567 | -1.261 | 1728 | 3695 | -0.686 |
| CLJU_c19480 | putative flavin reductase-like protein with rubredoxin domain | 1035 | 37947 | -4.767 | 796 | 5290 | -2.322 |
| CLJU_c19490 | ferritin | 1607 | 68688 | -4.988 | 1004 | 8844 | -2.727 |
| CLJU_c20050 | predicted MoaD/ThiS domain protein | 370 | 579 | -0.219 | 219 | 4871 | -4.063 |
| CLJU_c20060 | predicted dinucleotide-utilizing enzymes involved in molybdopterin/thiamine biosynthesis | 235 | 321 | -0.022 | 223 | 2703 | -3.186 |
| CLJU_c23080 | predicted hydrogenase expression/formation protein | 5433 | 7215 | 0.020 | 3775 | 25025 | -2.319 |
| CLJU_c35550 | predicted transcr. regulator, Fur family | 25077 | 201453 | -2.577 | 27102 | 104436 | -1.535 |
| CLJU_c37670 | CODH | 119541 | 101616 | 0.664 | 23751 | 183015 | -2.535 |

† Comparisons in gene expression were made between anaerobic and 8% $O_2$ exposed cells. A positive N.Log2 F.C. (normalized Log2 fold change) value reflects higher expression by anaerobic cells and a negative N.Log2 F.C. value reflects higher expression 8% $O_2$ exposed cells. The threshold for comparison was set at an N.Log2 F.C. value of +/- 2.