ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

# Physiological Response of *Clostridium carboxidivorans* During Conversion of Synthesis Gas to Solvents in a Gas-Fed Bioreactor

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**ABSTRACT:** *Clostridium carboxidivorans* P7 is one of three microbial catalysts capable of fermenting synthesis gas (mainly CO, $CO_2$, and $H_2$) to produce the liquid biofuels ethanol and butanol. Gasification of feedstocks to produce synthesis gas (syngas), followed by microbial conversion to solvents, greatly expands the diversity of suitable feedstocks that can be used for biofuel production beyond commonly used food and energy crops to include agricultural, industrial, and municipal waste streams. *C. carboxidivorans* P7 uses a variation of the classic Wood–Ljungdahl pathway, identified through genome sequence-enabled approaches but only limited direct metabolic analyses. As a result, little is known about gene expression and enzyme activities during solvent production. In this study, we measured cell growth, gene expression, enzyme activity, and product formation in autotrophic batch cultures continuously fed a synthetic syngas mixture. These cultures exhibited an initial phase of growth, followed by acidogenesis that resulted in a reduction in pH. After cessation of growth, solventogenesis occurred, pH increased and maximum concentrations of acetate (41 mM), butyrate (1.4 mM), ethanol (61 mM), and butanol (7.1 mM) were achieved. Enzyme activities were highest during the growth phase, but expression of carbon monoxide dehydrogenase (CODH), Fe-only hydrogenases and two tandem bi-functional acetaldehyde/alcohol dehydrogenases were highest during specific stages of solventogenesis. Several amino acid substitutions between the tandem acetaldehyde/alcohol dehydrogenases and the differential expression of their genes suggest that they may have different roles during solvent formation. The data presented here provide a link between the expression of key enzymes, their measured activities and solvent production by *C. carboxidivorans* P7. This research also identifies potential targets for metabolic engineering efforts designed to produce higher amounts of ethanol or butanol from syngas.

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**KEYWORDS:** *Clostridium*; solventogenesis; bioreactor; enzyme activity; gene expression

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## Introduction

Humankind began to exploit microbial fermentation to produce ethanol before 10,000 B.C. (Patrick, 1952) and the commercial production of butanol over 100 years ago (Dürre, 1998). The increasing demand for renewable, carbon-neutral liquid transportation fuels has intensified the study of their production through microbial fermentation of cellulosic feedstocks. Initially, the focus was solely on ethanol, which can either be a stand-alone fuel or act as a substitute for the gasoline supplement MTBE to reduce CO and $NO_x$ emissions (reviewed in Ahmed and Lewis, 2007; Henstra et al., 2007; Shaw et al., 2008). The hygroscopic nature and low caloric content of ethanol limits its use with current infrastructure. Butanol as a biofuel has the benefits of being less hygroscopic and possessing a higher caloric content than ethanol (Wallner et al., 2009). The fermentation of cellulosic feedstocks by acetogenic clostridia has been developed to produce both ethanol and butanol as commercially viable end products and has been studied extensively (Carroll and Somerville, 2009; Dürre, 2005; Grethlein et al., 1991; Weizmann and Rosenfeld, 1937).

The most studied approach for the production of renewable liquid biofuels is by direct fermentation of sugars extracted from food or energy crops. Direct fermentation requires pretreatment of feedstocks to convert carbohydrate polymers to sugars, followed by fermentation to desired end products. In contrast, indirect fermentation consists of the nearly complete and indiscriminant conversion of a wide variety of carbonaceous compounds to synthesis gas (syngas; largely CO, $CO_2$, and $H_2$) through gasification, followed by fermentation of this syngas to valuable end products (Datar et al., 2004; Henstra et al., 2007; Lewis et al., 2008; Tanner, 2008). An advantage of indirect fermentation is that syngas, and therefore end products like ethanol and butanol, can be produced from a wide variety of sources such as natural gas, coal, oil, biomass, municipal waste, and potentially even from the solar conversion of $CO_2$ (Fernandez et al., 2008; Pena et al., 1996; Service, 2009).

Syngas-fermenting microbial catalysts are a unique and critically important component of indirect fermentation. Many microorganisms are able to ferment syngas as a sole energy and carbon source (Oelgeschlager and Rother, 2008), but few microorganisms are known that can use syngas to produce biofuels. *Clostridium carboxidivorans* P7 (ATCC PTA-7827) is one of only nine bacteria known to produce solvents from syngas (reviewed in Köpke et al., 2011) and, along with "*Butyribacterium methylotrophicum*" (Lynd et al., 1982) and "*Clostridium ragsdalei*" (Maddipati et al., 2011), is also capable of producing butanol. Metabolic and genomic analyses indicate that *C. carboxidivorans* uses a variation of the Wood–Ljungdahl (acetyl-CoA) pathway to utilize syngas as the sole carbon and energy source to produce ethanol, butanol, acetate, and butyric acid as end products (Fig. 1) (Bruant et al., 2010; Hemme et al., 2010; Liou et al., 2005). The overall reactions for ethanol and acetic acid production from $H_2$ and CO are (Vega et al., 1989) and the change in Gibbs free energy at 298°K and 100 kPa are displayed below:

$$6CO + 3H_2O \rightarrow C_2H_5OH + 4CO_2 \qquad \Delta G^\circ = -217.4\,\text{kJ/mol} \tag{1}$$

$$6H_2 + 2CO_2 \rightarrow C_2H_5OH + 3H_2O \qquad \Delta G^\circ = -97.0\,\text{kJ/mol} \tag{2}$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \qquad \Delta G^\circ = -154.6\,\text{kJ/mol} \tag{3}$$

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O_2 \qquad \Delta G^\circ = -74.3\,\text{kJ/mol} \tag{4}$$

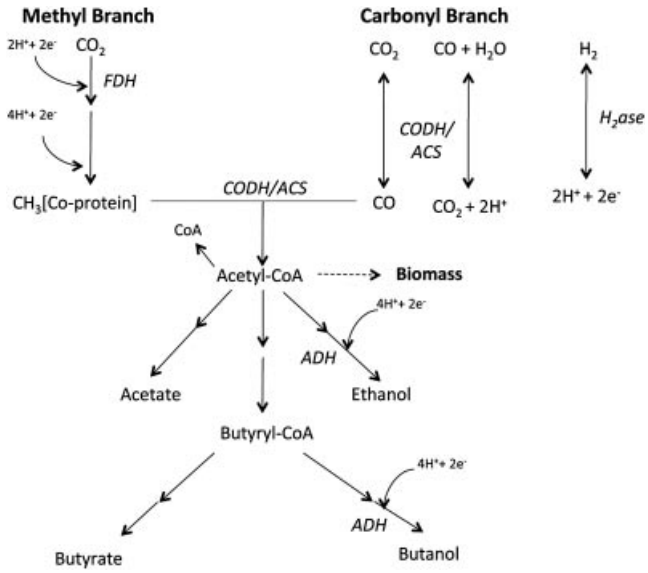


**Figure 1.** Conversion of CO and $CO_2$ to ethanol and butanol via the Wood–Ljungdahl pathway. Key metabolic products and flow of protons have been emphasized. The locations of key enzymes are shown: Alcohol dehydrogenase (ADH), formate dehydrogenase (FDH), carbon monoxide dehydrogenase/acetyl-CoA synthetase (CODH/ACS), and hydrogenase ($H_2$ase).

The overall reactions for butanol and butyric acid production from $H_2$ and CO are:

$$12CO + 5H_2O \rightarrow C_4H_9OH + 8CO_2 \qquad \Delta G^\circ = -486.4\,\text{kJ/mol} \tag{5}$$

$$12H_2 + 4CO_2 \rightarrow C_4H_9OH + 7H_2O \qquad \Delta G^\circ = -245.6\,\text{kJ/mol} \tag{6}$$

$$10CO + 4H_2O \rightarrow CH_3(CH_2)_2COOH + 6CO_2 \qquad \Delta G^\circ = -420.8\,\text{kJ/mol} \tag{7}$$

$$10H_2 + 4CO_2 \rightarrow CH_3(CH_2)_2COOH + 6H_2O \qquad \Delta G^\circ = -220.2\,\text{kJ/mol} \tag{8}$$

The purpose of this study was to provide a comprehensive physiological characterization of *C. carboxidivorans* growing under conditions analogous to those that would be used for industrial production of biofuels through indirect fermentation of cellulosic feedstocks. *C. carboxidivorans* was grown in a batch reactor that was continuously fed a synthetic syngas mixture. Product formation, the expression of genes for key metabolic enzymes, and their enzymatic activities were monitored at various time points. Of

particular interest was the expression of two acetaldehyde/alcohol dehydrogenases that are found in tandem on the genome (Bruant et al., 2010; Hemme et al., 2010). Genome analyses have only found this arrangement in one other syngas utilizing, solvent-producing acetogen, *Clostridium ljungdahlii* (Köpke et al., 2010). Our results show a difference in expression levels of these two genes by *C. carboxidivorans*.

## Materials and Methods

### Bioreactor Conditions and Sampling

Starter cultures of *C. carboxidivorans* P7 (ATCC PTA-7827) (Liou et al., 2005) were grown and maintained anaerobically in 250 mL serum seal bottles containing 100 mL of a modified basal medium (Liou et al., 2005; Tanner, 2007), with a syngas atmosphere ($N_2$:CO:$CO_2$:$H_2$ [60:20:15:5]) at 210 kPa, 37°C and pH 5.7. This medium was composed of ($L^{-1}$): Yeast extract, 1.0 g; mineral solution, 25 mL; trace metal solution, 10 mL; vitamin solution, 10 mL; MES, 10 g; resazurin, 1 mL; cysteine sulfide, 5 mL (Tanner, 2007). Under strict anaerobic conditions, aliquots (60 mL) from each starter culture were pooled (300 mL total, 10% v/v inoculum) and used to inoculate an unpressurized anoxic bioreactor (BioFlo 110, New Brunswick Scientific, Edison, NJ,) (7.5 L total volume) containing 3.0 L of basal medium. Agitation was provided by three six-blade Rushton turbine impellers, with an agitation speed set at 150 rpm. The temperature in the fermentor was maintained at 37°C. Four baffles were symmetrically arranged to avoid vortex formation of liquid media and improve mixing. A microsparger (New Brunswick Scientific) with a pore size of 10–15 μm was used for sparging a syngas mix ($N_2$:CO:$CO_2$:$H_2$ [60:20:15:5]; Stillwater Steel and Supply Co., Stillwater, OK) at a rate of 0.10 standard liter $min^{-1}$ (SLM). The syngas mix flowing out of the bioreactor passed through a condenser cooled at 5°C using a refrigerated circulator (1156 D, VWR International, West Chester, PA). Condensed vapor was returned to the fermentor to minimize losses of alcohols by evaporation and gas stripping. BioCommand software was used for data acquisition, monitoring, and controlling the fermentor. The initial pH of the medium was adjusted to 5.7 using 5N NaOH. The pH of the bioreactor culture was monitored using an integrated probe. Each of three sequential bioreactor runs was maintained until no increase in solvent production was observed. Every 24 h, a 40 mL liquid sample was taken to monitor pH, growth, and physiological parameters. Culture growth was measured from a 1 mL subsample as optical density (OD) at 660 nm wavelength with a 1 cm light path using a UV-vis spectrophotometer (Cary 50Bio, Agilent Technologies, Wilmington, DE). Fermentation products, enzyme activity, and gene expression were measured as described below.

### Fermentation Products

Fermentation products were analyzed for each of three bioreactor replicates from liquid subsamples (1 mL) every 24 h using methods described previously (Ramachandriya et al., 2010). Briefly, the samples were centrifuged at 10,000*g* for 10 min at room temperature and the supernatant was filtered with a 0.45 μm nylon membrane syringe filter (VWR International). Filtrates were analyzed for ethanol, acetic acid, butanol. and butyric acid with a gas chromatograph (GC) (6890 N, Agilent Technologies) fitted with a flame ionization detector (FID) and a J&W DB-FFAP chromatography column (Agilent Technologies). Hydrogen was the carrier gas at initial flow rate 1.9 mL $min^{-1}$ for 3.0 min and then the flow was increased to 4 mL $min^{-1}$ at a ramping rate of 0.5 mL $min^{-2}$. The inlet temperature was 200°C with a split ratio of 50:1. The oven temperature was held at 40°C for 1.5 min and then increased at a ramping rate of 40°C $min^{-1}$ to 235°C. The FID temperature was 250°C with $H_2$ (40 mL $min^{-1}$) and air (450 mL $min^{-1}$). The data were analyzed using CHEMSTATION® software (Agilent Technologies).

### Enzyme Activity

Enzyme assays were conducted for the third bioreactor run with whole cells collected from 20 mL subsamples (2,200 × g, 10 min, 4°C) and resuspended in 5 mL TAPS buffer (5.0 mM N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid, 2 mM $MgCl_2.6H_2O$ and 2 mM 2-mercaptoethanol; pH 8.5 with KOH) under strict anoxic conditions. Enzyme activities were measured using the substrate-dependent reduction of methyl viologen (MV) in stoppered, crimped anaerobic tubes (Bellco Glass, Vineland, NJ) at 40°C with a headspace of 100% $N_2$ (Balch and Wolfe, 1976; Saxena and Tanner, 2011). Resuspended cells (200 μL) were added to an anaerobic tube containing TAPS buffer with 4 mM MV to initiate each assay. Assays for ethanol dehydrogenase (EDH) and butanol dehydrogenase (BDH) activities contained 300 mM ethanol or 192 mM butanol, respectively. Assays for carbon monoxide dehydrogenase (CODH) or hydrogenase ($H_2$ase) activity contained TAPS buffer with 4 mM MV, but the nitrogen headspace was replaced with the substrate gases CO or $H_2$ at 160 kPa, respectively. The reduction of MV was monitored over time as absorbance at 578 nm (extinction coefficient for MV $\varepsilon_{578} = 9.78\ cm^{-1}\ mM^{-1}$) (Champine and Goodwin, 1991) at 40°C using a Spectronic® 20D+ (Thermo Scientific, Waltham, MA). One unit was defined as the quantity of enzyme that catalyzed the reduction of 2 μmol MV $min^{-1}$ (2 $e^-$ reduction) $mg^{-1}$ of protein (Saxena and Tanner, 2011). Protein content was measured using the bichinchoninic acid method (Smith et al., 1985) and bovine serum albumin was used as the standard. Cell mass concentrations were calculated from OD using the equation cell mass concentration at $t_n = 0.34 \cdot OD_{tn}$ (Maddipati et al., 2011). Statistical significance was determined for comparisons at

different time points using 2-tailed, unpaired Student's *t*-tests, with Walsh correction as needed (GraphPad InStat, Graph Pad Software, San Diego, CA).

### Gene Expression

For the third bioreactor run (Fig. 3), gene expression was monitored over time using reverse transcriptase quantitative PCR (RT-qPCR) with primers specific for the catalytic subunit of carbon monoxide dehydrogenase (*codh*, EC:1.2.99.2, Ccarb_0164), two tandem acetaldehyde/alcohol dehydrogenases (*adhE1* and *adhE2*; Ccarb_4321 and 4322) and two Fe-only hydrogenases (large subunit domain, Ccarb_2646, and 4385). Each primer pair (Table I) was developed manually from the *C. carboxidivorans* P7 draft genome sequence (JGI-PGF Project ID: 33115) (Hemme et al., 2010) and checked for $T_m$, hairpins, self-dimerization, and hetero-dimerization using the Oligo Analyzer software provided on the Integrated DNA Technologies website (Integrated DNA Technologies, Coralville, IA; http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Optimal conditions (primer concentrations and annealing temperatures) for each primer pair were identified as those producing the largest amount of product with no non-specific bands or primer-dimer, based on visualization on agarose gels.

At each time point, a 5 mL subsample was treated with 2.5 mL of RNA Protect® (Qiagen, Valencia, CA) and stored at −80°C until assayed. RNA was extracted from the frozen subsamples using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol with modifications. Cells were treated with lysozyme for 30 min at 25°C prior to mechanical lysis conducted with 0.1 mm glass beads (RPI Research, Mount Prospect, IL) shaking in a Mini-Beadbeater-8® (BioSpec Products, Inc., Bartlesville, OK) at maximum speed (2,800 oscillations $min^{-1}$, with a displacement of 3.18 cm) for 2 min at room temperature. DNA in each sample was digested using RNase-free DNase (Qiagen) while nucleic acids were still bound to the chromatography column, and again after elution using RQ1® RNase–free DNase (Promega, Madison, WI) according to manufacturer's protocols. Quantity and purity of nucleic acids was determined by absorbance spectra between 200 and 300 nm on a NanoPhotometer (Implen GmbH, Munich, Germany). Samples were deemed "DNA-free" if PCR with 16S rDNA primers gave no visible amplified product on an agarose gel.

Gene expression was measured using a two-step qRT-PCR. Following denaturation at 92°C, complementary DNA (cDNA) copies of mRNA for each gene were produced from 5 μL of RNA extract with m-MLV reverse transcriptase (Fisher Scientific, Pittsburg, PA) and gene-specific primers (0.2 nM) in a total reaction volume of 25 μL at 37°C for 72 min following manufacturer's protocols. Quantitative PCR was performed on triplicate 5 μL samples of the RT reaction product to determine the mean number of cDNA copies in each sample · $ng^{-1}$ of DNA using gene-specific primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in a total reaction volume of 30 μL. Thermal cycling and real time product detection was conducted in a 7300 Real-Time PCR system (Applied Biosystems) with the following conditions: 95°C for 10 min; 40 cycles (95°C for 45 s, 55°C for 45 s, and 72°C for 1 min). Ten-fold serial dilutions of *C. carboxidivorans* genomic DNA ($2.3–2.3 \times 10^4$ copies) were used as the standard for qPCR of *adh*1, *adh*2, and *codh* gene expression. For the Fe-only hydrogenase genes, PCR-amplified products cloned into *Escherichia coli* using the TOPA-TA cloning system for sequencing (Invitrogen, Carlsbad, CA) were used as standards. Each RT-qPCR experiment was visualized on an agarose gel to confirm the absence of non-specific products including primer-dimers. Statistical significance was determined for comparisons of gene expression at different time points and between different genes using 2-tailed, unpaired student's *t*-tests with Welsh correction as needed (GraphPad InStat, GraphPad Software).

## Results

### Growth and Fermentation Product Formation

The growth of *C. carboxidivorans* and the formation of fermentation products from three bioreactor replicates

**Table I.** Targeted genes and primers used in this study.

| Targeted gene[a] | Primer name | Sequence (5′–3′) | Amplicon size (bp) |
|---|---|---|---|
| Alcohol/acetaldehyde dehydrogenase 1, *adhE1* (Ccarb_4321) | adh1F | GAGAACCAACAGTTGAGTTATCTGGAA | 380 |
| | adh1R | TATAAGCAGGTTGTCCAACGA | |
| Alcohol/acetaldehyde dehydrogenase 2, *adhE2* (Ccarb_4322) | adh2F | TGTGCTTCAGAGCAGTCGGT | 482 |
| | adh2R | AATGTTCTACCAGTCTTCATAGTTTCTCTA | |
| Carbon monoxide dehydrogenase, *codh* (Ccarb_0164) | codhF | TGGTTGGATTTAGTACAGAGGCT | 285 |
| | codhR | GTCTTAAGTCCAGGACCAGCGAGT | |
| Fe-only hydrogenase, large subunit (Ccarb_2646) | hyd1F | GAGTATGCCCTACAGGTGCATTGG | 236 |
| | hyd1R | GATCTGTCCCACTGTTACTTTTGGTCC | |
| Fe-only hydrogenase, large subunit (Ccarb_4385) | hyd2F | TGGTGCTACAGGAGGAGTTATGGAAGC | 283 |
| | hyd2R | TTTTGGTTGACCGCCGCCAC | |

[a]Locus tags from *C. carboxidivorans* P7 draft genome (JGI-PGF Project ID: 33115) are given in parentheses.

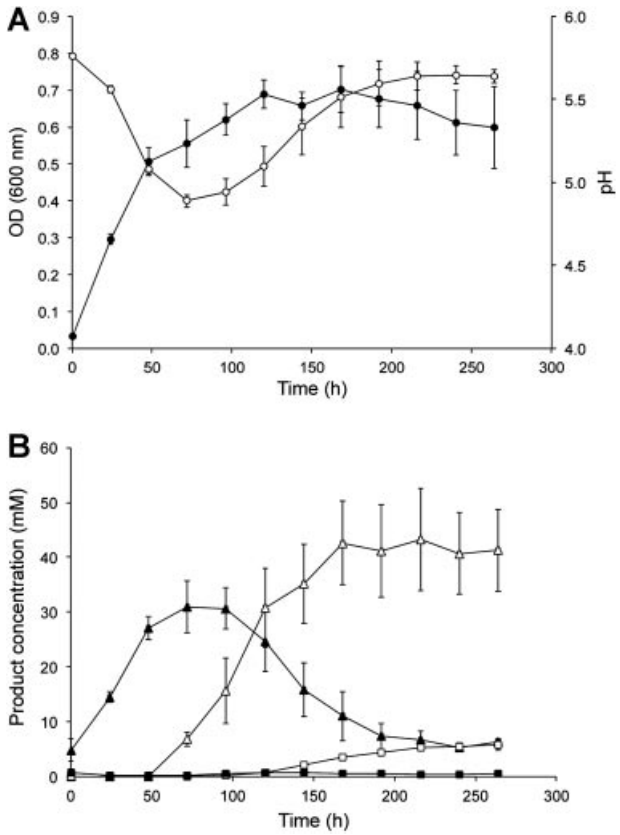


**Figure 2.** Mean values for (**A**) measured growth ($OD_{660}$, solid circles) and pH (open circles) and (**B**) product formation (acetate, solid triangles; butyrate, solid squares; ethanol, open triangles; and butanol, open squares) over time (h) from three syngas-fed bioreactor runs. Error bars represent standard error ($n = 3$).

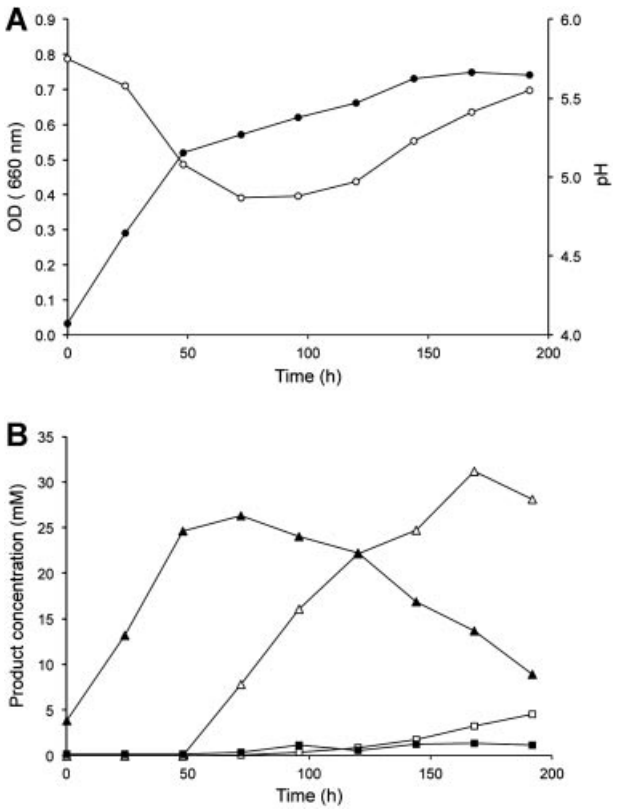


**Figure 3.** **A**: Measured growth ($OD_{660}$, solid circles) and pH (open circles) and (**B**) product formation (acetate, solid triangles; butyrate, solid squares; ethanol, open triangles; and butanol, open squares) over time (h) for the third bioreactor run.

followed a pattern common for acetogenic clostridia (Fig. 2A). Acetate accumulation (up to 31 mM) coincided with a decrease in pH and growth rate during the first 72 h. Initiation of solvent formation after 50 h coincided with a decrease in acetate and increase in pH. Among the bioreactor replicates, solvent production ranged from 32.1 to 61.2 mM ethanol and 4.4–7.1 mM butanol (Fig. 2B). Ethanol production appeared to increase at the expense of acetic acid, with no substantial increase in solvent production observed after 192 h. The rate of ethanol production peaked at 35–65 mM $g^{-1}$ of cells $day^{-1}$ between 72 and 96 h, and then dropped to less than 20 mM $g^{-1}$ of cells $day^{-1}$ for the rest of the experiment (data not shown). In contrast, butanol production was first detected after 96 h. As shown in Figure 3, each individual replicate began with a period of growth (10.2 h mean generation time) that began less than 24 h after inoculation.

## Key Enzymatic Activity

Whole cell enzyme assays were used to monitor enzyme activity over time within the third bioreactor replicate. $H_2$ase activity was highest during the first 50 h, followed by a sharp decline at 72 h and then a gradual increase throughout the remainder of the experiment (Fig. 4A). CODH showed much less activity than $H_2$ase, but followed a similar trend that reached a minimum at 96 h. Butanol and EDH activity was also greatest during cell growth, reaching a minimum at 96 h (Fig. 4B). BDH activity, however, was greater than EDH at all time points ($P < 0.05$) except during the transition to solventogenesis between 72 and 96 h, and during late stationary phase at 192 h.

## Gene Expression for Key Enzymes

To enable direct comparison with enzyme activity measurements, the third bioreactor run was the source of all gene expression data. Both tandem acetaldehyde/alcohol dehydrogenase genes (*adh1* and *adh2*) were expressed during all stages of the bioreactor run, showing a large increase during solventogenesis (Fig. 5A). Expression of the *adh* genes was differential, with greater expression of *adh2* than *adh1*. Differential expression was nominal during the production of primarily ethanol (5.4 and 6.8-fold at 48 and 96 h), but increased significantly during butanol production (184.7-fold

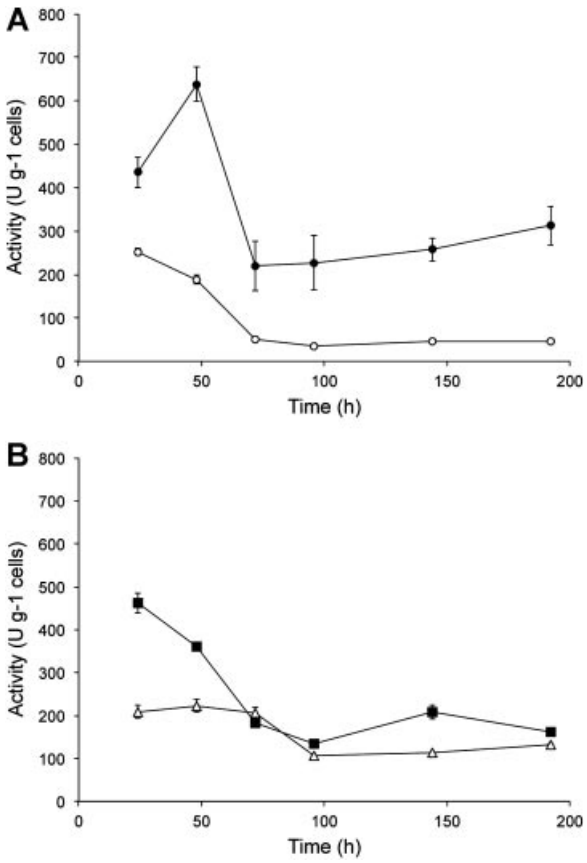


**Figure 4.** Measured activities of (**A**) hydrogenase (solid circles) and carbon monoxide dehydrogenase (open circles); (**B**) butanol dehydrogenase (closed squares) and ethanol dehydrogenase (open triangles) in units (U) per gram cells over time. Error bars represent standard deviation of mean values ($n = 3$).

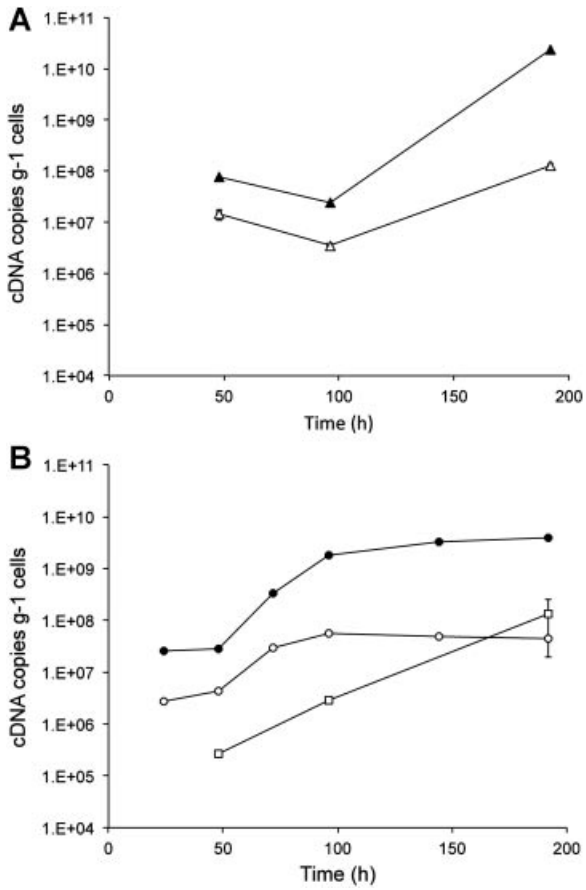


**Figure 5.** Gene expression of (**A**) *adh*1 (open triangles) and *adh*2 (closed triangles) and (**B**) codh (open squares), two Fe-only dehydrogenases (Ccarb_2646, closed circles; Ccarb_4385, open circles) measured as the number of cDNA copies per gram of cells over time (h). Error bars represent standard error of mean measurements ($n = 3$).

at 192 h) ($P < 0.01$). Expression of the catalytic subunit of carbon monoxide dehydrogenase (*codh*) increased over 6,000-fold from 48 to 192 h (Fig. 5B). Both Fe-only hydrogenase genes, Ccarb_2646 and Ccarb_4385, showed expression responses to changing culture conditions, increasing prior to 48 h and coinciding with the initiation of ethanol formation (Fig. 5B).

## Discussion

There are as few as nine acetogenic bacteria capable of autotrophic growth on CO, producing acetate and other products (Köpke et al., 2011). These microorganisms use CO, $CO_2$, and $H_2$ via the Wood–Ljungdahl (acetyl-CoA) pathway to produce acetyl-CoA, a precursor of cell biomass, acetate, ethanol, butyrate, butanol, and potentially many other products (Drake et al., 2008; Gössner et al., 2008). Only three of these acetogens, *Clostridium carboxidivorans* (Liou et al., 2005), "*Clostridium ragsdalei*" (Maddipati et al., 2011), and "*Butyribacterium methylotrophicum*" (Lynd et al., 1982) are known to be intrinsically capable of producing butanol.

In the work described here, growth, product formation, enzyme activity, and gene expression were monitored for the CO-fermenting acetogen *C. carboxidivorans* growing in a bioreactor continuously fed syngas as the sole source of carbon and energy. This research was carried out under growth conditions likely to be used in the industrial production of ethanol and butanol. A goal of this work was to link transcriptional control of key metabolic genes with their measured activities and product formation in a bioreactor.

### Cell Growth and Product Formation

Medium pH is believed to serve as a trigger causing acetogens to shift from the production of acids to their respective solvents (Dürre, 2005). The pH of the bioreactor was not controlled after the initial pH (5.7) adjustment

of the medium. As expected, all bioreactor replicates demonstrated a decrease in pH as acids were formed, followed by an increase in pH that coincided with the conversion of acids to alcohols (Fig. 2).

The highest measured growth rates during the initial stages of the bioreactor indicated generation times of 10.2 h, slower than the maximal doubling time of 4.3 h recorded for bottle cultures that were run with a different gas composition and at higher pressures (Liou et al., 2005). The gas compositions used in this study ($N_2$:CO:$CO_2$:$H_2$ [60:20:15:5]) was chosen to model syngas produced during gasification of switchgrass, which contains three times less CO than the gas used by Liou et al. (2005). Measured growth in the bioreactor was linear, never exponential, which was interpreted as an indication of substrate limitation due to mass transfer of the gaseous substrate to the aqueous phase. We are currently studying improved reactor designs to reduce mass transfer limitations. For example, using hollow fiber membranes as diffusers has recently been reported to improve the mass transfer of CO in water (Lee et al., 2012; Munasinghe and Khanal, 2012).

Two distinct rates of growth were observed in the bioreactor. The first, faster growth rate occurred during the first 48 h. The production of organic acids in the Wood–Ljungdhal pathway yields more ATP than when alcohols are produced (White, 2007). Along with a minimum pH (4.8–5.0) and a maximum acetate concentration (23–34 mM) for the three bioreactor runs, transition to a second, slower growth rate coincided with the production of the solvents ethanol and butanol.

## Enzyme Activity

The activity of several important enzymatic reactions was monitored throughout the third bioreactor run, which included the growth phase (first 48 h) and the stationary phase of the bioreactor. Activity for all measured enzymatic reactions peaked during the first 48 h, followed by significantly less activity (Fig. 4A and B). Despite the lower levels of EDH and BDH activity measured during solventogenesis, these levels were still well above those needed to produce the alcohols accumulated in the bioreactor. The difference between measured activity and actual rate of production suggests that product formation is limited by something other than the amount of active enzyme present in the cell. For example, the predicted rate of ethanol and butanol production based on activity measurements was higher than the actual rate of formation per day (data not shown). It is even more interesting that the rate of butanol formation over time was different from that of ethanol ($P < 0.05$). The rate of ethanol formation peaked by 120 h, whereas butanol formation gradually increased throughout the stationary phase and increase in pH, peaking only after 192 h. This was interpreted as a preference of *C. carboxidivorans* for ethanol formation compared to butanol formation.

A potential limitation of the enzyme activity measurements was that only the reverse enzyme reactions were measured. These measured activities may not be directly interpretable if the kinetics of these reactions were not equal in each direction (Gheshlaghi et al., 2009). Additionally, the enzyme assays were conducted with a saturating amount of substrate, which is likely to be higher than intracellular concentrations during growth in the bioreactor.

## Ethanol and Butanol Production

The onset of ethanol production around 48 h did not coincide with an appreciable increase in EDH enzyme activity. Instead, this activity was at its peak at around 48 h, but decreased by 50% between 72 and 96 h (Fig. 4B). Expression of the two tandem, bi-functional acetaldehyde/alcohol dehydrogenase genes (*adh*1 and *adh*2) also decreases by about the same amount between 48 and 96 h (Fig. 5A). Between 96 and 192 h, expression of both *adh* genes increased significantly ($P < 0.01$). Neither the EDH activity nor the rate of production of ethanol seemed to reflect the dynamics of gene expression measured for either gene. One possible interpretation for the large increase in expression is an attempt by the cell to compensate for a drop in efficiency of ethanol production. This and alternative explanations, however, remain untested.

The presence of two tandem, homologous *adh* genes has only been observed on the genomes of *C. carboxidivorans* P7 and *C. ljungdahlii*. Although both genes are annotated as acetaldehyde dehydrogenase (EC 1.2.1.10)/alcohol dehydrogenase *adhE* (EC 1.1.1.1), they are not identical. They differ in length by 15 bases (five amino acids) and share only 82% identity (695/847), or 91% allowing for conservative substitutions (775/847) at the amino acid level (Altschul et al., 1997). The 200 bp long region between these genes contains a putative terminator for *adh*1 followed by a promoter region for *adh*2. The genetic structure and genomic context of these two genes support the potential for differential expression, which was readily observed by RT-qPCR (Fig. 5A).

The *adh*2 gene, second in the tandem arrangement on the genome, was always expressed at a higher level than *adh*1 (Fig. 5A). More importantly, at some point after 96 h both genes are up-regulated but to very different degrees. The expression of *adh*1 increased 36-fold between 96 and 192 h, whereas, *adh*2 expression increased over 1,000-fold over the same time span. The period of greatest differential expression corresponded to the greatest rate of butanol production, raising questions as to the potential differential roles between these two enzymes.

## Energy Conservation

*C. carboxidivorans* was in a stationary phase of growth following the decrease in pH and production of solvents (Figs. 2 and 3). The oxidation of CO to $CO_2$, carried out by

CODH, generates energy captured by the reduction of ferredoxin (Ragsdale, 2004). The levels of CODH activity were greatest during the first 72 h of the bioreactor replicate (Fig. 4A), but the expression of *codh* increased over 500-fold from 48 to 192 h (Fig. 5B). This discrepancy again points to a potential for decreased efficiency of the enzyme. Additionally, the culture was still consuming CO but at a decreased rate compared to the first 72 h (data not shown).

$H_2$ase activity was greatest during the first 48 h, dropping threefold at 72 h, followed by a modest increase in measured activity until 192 h. Expression of the two Fe-only $H_2$ases monitored suggests that they were not responsible for the initial high amounts of activity but become more important after 48 h with increases in expression of 100-fold (Ccarb_2646) and 10-fold (Ccarb_4385) (Fig. 5B). If the CODH is not participating in the oxidation of CO to $CO_2$ via the biological water-gas shift reaction, the Fe-only hydrogenases may be required to compensate for the deficit in reducing equivalents (Ragsdale, 2004). Additionally, these $H_2$ases could be involved in generating a membrane gradient for the generation of ATP (Biegel and Müller, 2010; Köpke et al., 2010; Müller et al., 2008) via the Rnf complex found on its genome (Bruant et al., 2010; Hemme et al., 2010).

A strain of *C. ljungdahlii* transformed with the necessary genes was also capable of producing trace amounts of butanol from CO (Köpke et al., 2010). These microorganisms can act as microbial catalysts for the conversion of CO-containing gases into valuable fuels and chemicals. Industrial waste gases, and gasification of coal, biomass and municipal solid waste can all serve as sources of CO. The gasification of non-food lignocellulosic feedstocks (e.g., switchgrass, forestry waste) offers a promising approach to producing fuels and chemicals that are renewable and carbon-neutral.

The development of strategies to use gas fermentation for the commercial production of fuels and chemicals is on the rise, but not fully mature (Köpke et al., 2011). Significant developments have come from the optimization of nutrient concentrations and pH, which influence cellular growth, substrate efficiency, and the ratio of acids versus alcohols produced (Saxena and Tanner, 2011). Much is known about the enzymes, pathways and energetics of autotrophic, gas-fermenting acetogens (Gheshlaghi et al., 2009; Ljungdahl, 1986) but our understanding of the molecular processes underlying this biochemistry is limited. This study measured the expression of tandem *adh* genes during solvent formation, which suggests they may have different roles. Isolation and characterization of these *adh* genes may provide insight into the physiological regulation of syngas solventogenesis. The genetic information needed to identify the molecular underpinnings of this useful biochemistry has only recently become available with the release of genome sequences for *C. ljungdahlii* and *C. carboxidivorans* (Bruant et al., 2010; Hemme et al., 2010; Köpke et al., 2010) and is a prelude to exciting new research.

## Conclusions

Linking the production of syngas from various waste streams or renewable, non-food lignocellulosic feedstocks with subsequent fermentation to solvents and other useful products is a promising and maturing industrial process. At the heart of this process are the novel microbial catalysts that are capable of CO fermentation and product formation. Optimized media conditions and reactor designs have addressed some of the limitations, but further study of the metabolic pathways and their molecular underpinnings is paramount.

Several important observations are presented here that were made by simultaneously monitoring growth, product formation, and gene expression of *C. carboxidivorans* in a syngas-fed bioreactor. The increased expression of CODH and Fe-only $H_2$ases during later stages of the bioreactor fermentation implies their importance during solventogenesis and suggests a need for reducing equivalents. Additionally, the differential expression of two tandem *adh* genes suggests a differential role for each during solvent production. Genetic manipulation of these processes in *C. carboxidivorans* or through their transformation into other suitable microorganisms is likely to provide further optimization that will directly benefit our understanding of this useful biochemistry and its commercialization.

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