Deletion of Type I glutamine synthetase deregulates nitrogen metabolism and increases ethanol production in *Clostridium thermocellum*

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28   **Abstract**

29   *Clostridium thermocellum* rapidly deconstructs cellulose and ferments resulting
30   hydrolysis products into ethanol and other products, and is thus a promising
31   platform organism for the development of cellulosic biofuel production via
32   consolidated bioprocessing. While recent metabolic engineering strategies have
33   targeted eliminating canonical fermentation products (acetate, lactate, formate, and
34   H₂), *C. thermocellum* also secretes amino acids, which has limited ethanol yields in
35   engineered strains to approximately 70% of the theoretical maximum. To investigate
36   approaches to decrease amino acid secretion, we attempted to reduce ammonium
37   assimilation by deleting the Type I glutamine synthetase (*glnA*) in an essentially
38   wild type strain of *C. thermocellum*. Deletion of *glnA* reduced levels of secreted
39   valine and total amino acids by 53% and 44% respectively, and increased ethanol
40   yields by 53%. RNA-seq analysis revealed that genes encoding the RNF-complex
41   were more highly expressed in $\Delta glnA$ and may have a role in improving NADH-
42   availability for ethanol production. While a significant up-regulation of genes
43   involved in nitrogen assimilation and urea uptake suggested that deletion of *glnA*
44   induces a nitrogen starvation response, metabolomic analysis showed an increase in
45   intracellular glutamine levels indicative of nitrogen-rich conditions. We propose
46   that deletion of *glnA* causes deregulation of nitrogen metabolism, leading to
47   overexpression of nitrogen metabolism genes and, in turn, elevated glutamine
48   levels. Here we demonstrate that perturbation of nitrogen assimilation is a
49   promising strategy to redirect flux from the production of nitrogenous compounds
50   toward biofuels in *C. thermocellum*.

51

52 Key Words: Cellulosic ethanol, *Clostridium thermocellum*, glutamine synthetase,
53 amino acid secretion

54

55 **1. Introduction**

56 Increasing global energy demands, depletion of low extraction-cost fossil fuel
57 reserves, and environmental concerns are driving forces for the search for renewable
58 and environmentally friendly energy sources. Bioethanol is among the leading
59 alternatives to petroleum-derived fossil fuels for the transportation sector given that
60 it is compatible with current engine technologies, burns cleaner than gasoline, has a
61 high octane/cetane number, and can be derived from renewable biomass feedstocks
62 (Arora et al., 2015; Vohra et al., 2014). The USA and Brazil are among the leaders
63 in ethanol production, generating 14.8 and 7.1 billions of gallons of ethanol in 2015,
64 respectively (RFA, 2016). Of the total global bioethanol produced, roughly 50% is
65 derived from maize and 30% is derived from sugarcane (DEFRA, 2008). This
66 dependence of bioethanol production on food/feed crops may increase food costs
67 and volatility of ethanol prices (Vohra et al., 2014). Life cycle analysis has further
68 shown that substituting gasoline with cellulosic ethanol can reduced greenhouse gas
69 emission by up to 115%, versus a 19-48% reduction with corn ethanol (Wang et al.,
70 2012). While the 2005 USA Energy Policy Act mandates an increase of biofuel
71 production from 22 to 36 billion gallons per year by 2022, the cap on starch-derived
72 ethanol is to remain at 15 billion gallons per year (RFA, 2016). Thus, a transition to
73 lignocellulosic feedstocks for bioethanol production is needed.

74

75 Currently, bioethanol is predominantly made via fermentation of mono- or
76 disaccharides using *Saccharomyces cerevisiae* or *Zymomonas mobilis*. While these
77 organisms are capable of high ethanol yields and titers, they cannot natively
78 deconstruct highly recalcitrant lignocellulosic biomass to liberate fermentable sugars
79 (Himmel et al., 2007). Thus, lignocellulosic bioethanol production using these
80 organisms requires chemical and/or enzymatic hydrolysis of lignocellulosic
81 biomass, separate cellulase production, and subsequent fermentation, increasing
82 production costs. Alternatively, a number of organisms are capable of cellulase-
83 mediated cellulose hydrolysis and subsequent fermentation to ethanol via
84 consolidated bioprocessing (CBP), potentially allowing for enhanced process
85 economics (Lynd et al., 2005; Lynd et al., 2002). However, to date, no stand-alone
86 process can produce ethanol from lignocellulose at yields and titers sufficient for
87 economic viability.

88

89 *Clostridium thermocellum* is a promising candidate for lignocellulosic bioethanol
90 production via CBP because it has the innate ability to rapidly solubilize raw
91 biomass with minimal or no pre-treatment (Paye et al., 2015), and ferment resulting
92 cellulosic hydrolysis products into ethanol. However, due to branched product
93 pathways that divert carbon and electrons to products other than ethanol, wild type
94 *C. thermocellum* ethanol yields are approximately 30% of the theoretical maximum
95 of 2 moles ethanol per mole hexose, well below industrial requirement of at least
96 90%. In addition to canonical fermentation products (acetate, lactate, formate, and
97 H₂), secreted amino acids (primarily valine and alanine) (Ellis et al., 2012;

98 Kridelbaugh et al., 2013; van der Veen et al., 2013) and other compounds including
 99 pyruvate, malate, fumarate, isobutanol, and butanediol (Holwerda et al., 2014),
 100 further limit ethanol yields. Metabolic pathways have been elucidated via
 101 enzymology (Carere et al., 2014; Lamed and Zeikus, 1980; Lin et al., 1998; Rydzak
 102 et al., 2009; Sparling et al., 2006; Taillefer et al., 2015; Zhou et al., 2013),
 103 transcriptomics (Carere et al., 2014; Deng et al., 2013; Raman et al., 2011; Wilson et
 104 al., 2013; Yang et al., 2012), proteomics (Raman et al., 2009; Rydzak et al., 2014;
 105 Rydzak et al., 2012), and genetics (Argyros et al., 2011; Biswas et al., 2014; Biswas
 106 et al., 2015; Deng et al., 2013; Tripathi et al., 2010). Furthermore, improvements in
 107 *C. thermocellum* electrotransformation protocols (Olson and Lynd, 2012; Tyurin et
 108 al., 2004), transformation efficiencies (Guss et al., 2012), genetic modification
 109 methods (Argyros et al., 2011; Mohr et al., 2013; Tripathi et al., 2010), and tools for
 110 heterologous expression (Groom et al., 2016; Olson et al., 2015) have allowed for
 111 rational metabolic engineering of *C. thermocellum* to improve ethanol yields by
 112 eliminating competing pathways and expressing heterologous ones. While deletion
 113 of phosphotransacetylase (*pta*) (Tripathi et al., 2010) and pyruvate:formate lyase
 114 (*pfl*) (Rydzak et al., 2015) eliminated acetate and formate production, respectively,
 115 marginal impacts on ethanol production were observed. Alternatively, elimination
 116 of lactate, lactate and acetate, or H₂ via deletion of lactate dehydrogenase (*ldh*)
 117 (Biswas et al., 2014), *ldh* and *pta* (Argyros et al., 2011; van der Veen et al., 2013),
 118 or [FeFe] hydrogenase maturation factor (*hydG*) and [NiFe] Ech-type hydrogenase
 119 (*echA-F*) (Biswas et al., 2015), respectively, improved ethanol production. While
 120 stacking all of these deletions except *echA-F* in a single strain ($\Delta hydG \Delta ldh \Delta pfl$

121 *Δpta-ack*; strain AG553) resulted in the highest ethanol yielding *C. thermocellum*
122 strain to date (Papanek et al., 2015), ethanol yields typically ranged from 50-70% of
123 the theoretical maximum, which is still below the benchmark 96% ethanol yields
124 achieved by *Saccharomyces cerevisiae* or *Zymomonas mobilis* (Panesar et al., 2006).
125 Recently strain AG553 was evolved to grow faster, and the resulting strain was able
126 to produce 22 g/L (478 mM) ethanol at 75% of the theoretical maximum yield on 60
127 g/l cellulose (Tian et al., 2016).

128
129 Recent studies have demonstrated that secreted amino acids typically account for
130 approximately 4-10% of total substrate consumed in wild type *C. thermocellum*,
131 reaching as high as 17% in certain mutants (i.e. evolved *Δldh Δpta* strain), with
132 valine and alanine accounting for the bulk of these amino acids (Ellis et al., 2012;
133 Holwerda et al., 2014; Kridelbaugh et al., 2013; van der Veen et al., 2013). The
134 extent of amino acid secretion has also been shown to be dependent on medium
135 nitrogen content (Kridelbaugh et al., 2013) and carbon loading (Holwerda et al.,
136 2014), and has been proposed to alleviate carbon and electron imbalances. While
137 few studies have measured amino acid secretion in other cellulolytic organisms, free
138 amino acids in *Clostridium cellulolyticum* fermentations have been reported to
139 account for 15% and 6% of total carbon consumed on cellobiose and cellulose,
140 respectively (Desvaux et al., 2001a; Desvaux et al., 2001b; Desvaux et al., 2001c;
141 Guedon et al., 1999), demonstrating that amino acid secretion in cellulolytic bacteria
142 is not exclusive to *C. thermocellum*. In *C. cellulolyticum*, 30-fold higher

143 protein/DNA ratios in supernatants versus cell extracts suggest that cell lysis is not
144 the cause of amino acids in the medium.

145

146 In most bacteria, ammonium assimilation typically occurs via glutamate
147 dehydrogenase (GLDH) or through the combination of glutamine synthetase (GS)
148 and glutamine-oxoglutarate aminotransferase/glutamate synthase (GOGAT).
149 GLDH catalyzes the reductive amination of α -ketoglutarate to glutamate using
150 NAD(P)H, typically under nitrogen-rich conditions (Shimizu, 2013). Alternatively,
151 under nitrogen-limited conditions, ATP-dependent GS aminates glutamate to
152 glutamine, and NAD(P)H or ferredoxin-dependent GOGAT replenishes the
153 glutamate pool using glutamine and α -ketoglutarate. GSs can be divided into three
154 groups which can be typically distinguished by amino acid length. These include
155 Type I GSs (~360 aa) which are widely distributed among bacteria, Type II GSs
156 (~450 aa) which are typically found in plants and some soil bacteria, and Type III
157 GSs (~730 aa) which have been identified in anaerobic bacteria and cyanobacteria
158 (van Rooyen et al., 2011). While the structures and multimeric assembly of GSs
159 vary among these classes, differences in function, if any, still remains unclear.

160 With the exception of one study nearly three decades ago (Bogdahn and Kleiner,
161 1986), little has been done to elucidate nitrogen assimilation in *C. thermocellum*.
162 Enzyme activity studies showed high NADPH-dependent GLDH and moderate GS
163 activities, but failed to detect any NADH or NADPH-dependent GOGAT activity
164 (Bogdahn and Kleiner, 1986). Given that glutamate and glutamine are the primary

165 source of assimilated nitrogen for the synthesis of amino acids, we hypothesized that
166 limiting the rate of nitrogen assimilation via glutamate and/or glutamine could
167 decrease total amino acid production, and in turn decrease levels of secreted amino
168 acids in *C. thermocellum*. Here, we deleted *glnA* (Clo1313_2031) encoding the
169 Type I GS in an attempt to redirect carbon and electron flux away from amino acids
170 towards more canonical fermentation products including ethanol.

171

172 **2. Materials and Methods**

173 *2.1. Plasmid and strain construction*

174 All enzymes and reagents used for cloning were purchased from New England
175 Biolabs (Ipswich, MA), unless otherwise specified. *Escherichia coli* Top10 (*dam*⁺
176 *dcm*⁺; Invitrogen, Carlsbad, CA) and BL21 (*dam*⁺ *dcm*⁻; New England Biolabs,
177 Ipswich, MA), used for plasmid construction and propagation, respectively, were
178 grown aerobically on LB medium supplemented with 12 µg/ml chloramphenicol as
179 required for plasmid maintenance. Plasmid isolation and purification was
180 performed using QIAprep spin miniprep kits (QIAGEN, Germantown, MD).
181 Plasmid pNJ22::2031_del for deletion of *glnA* (Clo1313_2031) was constructed
182 using Gibson Assembly (Gibson 2009) according to the manufacturer's guidelines
183 using primers provided in Supplementary File 1. Briefly, fragments corresponding
184 to regions 500 bp upstream, downstream, and within the first 500 bp of
185 Clo1313_2031 coding region, and a 1388 bp fragment encoding a chloramphenicol
186 acetyltransferase (*cat*) and a hypoxanthine phosphoribosyltransferase (*hpt*) driven by

187 a *C. thermocellum* glyceraldehyde-3-phosphate (GapDH) promoter were amplified
188 using Phusion polymerase (New England Biolabs, Ipswich, MA). Resulting PCR
189 fragments were treated with DpnI to digest residual template DNA and were ligated
190 with EcoRV digested pNJ022 using Gibson assembly generating pNJ22::2031_del
191 (Supplementary File 2). Correct assembly of fragments was verified using
192 restriction digests and sequencing using primers provided in Supplementary File 1.
193 All plasmids used for *C. thermocellum* transformations were ultimately isolated
194 from *E. coli dam⁺ dcm⁻* BL21 (Guss et al., 2012).
195
196 *Clostridium thermocellum* DSM 1313 (Deutsche Sammlung von Microorganismen
197 und Zellkulturen, Braunschweig, Germany) derivatized strains (Δhpt and $\Delta hpt\Delta glnA$)
198 were routinely grown anaerobically at 55°C, unless otherwise specified, in a Coy
199 anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) on 5 g/l cellobiose
200 in CTFUD medium (Rydzak et al., 2015). Medium was made anaerobic via
201 autoclaving to remove O₂ from solution, followed by immediate transfer to the
202 anaerobic chamber to maintain anaerobicity. Medium was supplemented with 10
203 µg/ml thiamphenicol, 50 µg/ml 5-fluoro-2'-deoxyuridine, or 500 µg/ml 8-
204 azahypoxanthine (Tokyo Chemical Industry, Co., Tokyo, Japan) during *C.*
205 *thermocellum* strain construction when appropriate. All selection steps were
206 performed at 50°C. *C. thermocellum* Δhpt (Argyros et al., 2011) was transformed
207 via electroporation as previously described (Guss et al., 2012; Olson and Lynd,
208 2012). This strain allows the *hpt* gene to be used as a counter-selectable marker to
209 enable gene deletion. The *glnA* gene was deleted as previously described (Argyros

et al., 2011; Olson and Lynd, 2012) using plasmid pNJ22::2031_del. Gene deletion was confirmed using primer sets provided in Supplementary File 1 as shown in Supplementary File 3. Deletion of *glnA* was further confirmed via whole genome resequencing and RNA-seq analysis (discussed below). Culture purity was routinely confirmed via 16S sequencing. This resulted in strain AG1329 (*C. thermocellum* $\Delta hpt\Delta glnA$). This whole process was repeated to delete *glnA* from *C. thermocellum* Δhpt a second time to recreate the strain to eliminate the possibility that secondary mutations are responsible for the phenotype, resulting in strain AG2070 (also *C. thermocellum* $\Delta hpt\Delta glnA$).

2.2. Fermentation conditions

Fermentation experiments were performed at 55°C in sealed, nitrogen sparged Balch tubes (27 ml; Belco Glass Inc., Vineland, NJ) containing 10 ml of MTC₅ medium supplemented with 4.5 g/l cellobiose as previously described (Rydzak et al., 2015). Tubes were inoculated with 2% exponential phase (OD₆₀₀ ~ 0.4) cultures grown on MTC₅. Final fermentation product, amino acid, biomass, and pH measurements were taken following complete cellobiose utilization (<1.0 mM remaining). Fermentations were performed a minimum of three times with three independent biological replicates each time. Samples for RNA-seq and metabolomic analysis were grown at 55°C in sealed, nitrogen sparged 162 ml serum bottles containing 50 ml of MTC₅ supplemented with 4.5 g/l cellobiose. Samples were harvested in early exponential phase (OD₆₀₀ ~0.33) and analysis was performed in quadruplicate for each strain. To monitor growth rates, cells were grown in 48 well microtiter plates

233 in quadruplicate (Corning, Tewksbury, MA) containing 600 μ l MTC₅ in an Eon
234 Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT) situated in
235 a Coy anaerobic chamber. All chemicals were reagent grade and obtained from
236 Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA) unless
237 otherwise specified.

238

239 *2.3. Analytical measurements*

240 Cell growth was monitored spectrophotometrically at OD₆₀₀ in an Eon Microplate
241 Spectrophotometer (BioTek Instruments Inc., Winooski, VT). Cellobiose, glucose,
242 and fermentation products (pyruvate, lactate, acetate, formate, ethanol) were
243 analyzed using a Breeze High Performance Liquid Chromatography system (Waters,
244 Milford, MA) using an Aminex-HPX-87H column (Bio-Rad, Hercules, CA) with a
245 5 mM sulfuric acid mobile phase. H₂ was measured using an Agilent Technologies
246 6850 Series II Gas Chromatograph (Agilent Technologies, Santa Clara, CA) using a
247 thermoconductivity detector set at 190°C with a N₂ reference flow and a Carbonex
248 1010 PLOT (30.0 m $\times$ 530 μ m I.D.; model Supelco 25467) column. Secreted amino
249 acids were measured using an Aracus Amino Acid Analyzer (membraPure, Berlin,
250 Germany) using a T111 Li-cation exchange column with eluents supplied by the
251 manufacturer. Ninhydrin derived amino acids were measured photometrically at
252 570 nm with the exception of proline, which was measured at 440 nm. Final pH
253 was measured using an Accumet AB15 Basic pH meter (Fisher Scientific;
254 Pittsburg, PA) at room temperature and in room atmosphere. Total nitrogen and
255 carbon in cell pellets was were determined using a Simadzu TOC-Vcph Total

256 Organic Carbon analyzer with added Total Nitrogen unit and a ASI-V autosampler
257 (Shimadzu Scientific Instruments, Columbia, MD) with an Avicel and glycine
258 standards for carbon and nitrogen determination, respectively (Holwerda et al.,
259 2012).

260

261 *2.4. Whole genome resequencing*

262 Genome resequencing and analysis was performed by the DOE Joint Genome
263 Institute (JGI, Walnut Creek, CA) with an Illumina MiSeq instrument. Genomic
264 DNA was extracted using a Qiagen DNeasy kit (Qiagen, Valencia, CA). 100 ng of
265 DNA was sheared to 500 bp fragments using the Covaris LE220 ultrasonicator
266 (Covaris) and size selected using AMPure XP SPRI beads (Beckman Coulter). The
267 fragments were treated with end-repair, A-tailing, and ligation of Illumina
268 compatible adapters (IDT, Inc) using the KAPA-Illumina library creation kit
269 (KAPA Biosystems). The prepared libraries were quantified using KAPA
270 Biosystem's next-generation sequencing library qPCR kit and run on a Roche
271 LightCycler 480 real-time PCR instrument. The quantified multiplexed libraries
272 were pooled in sets of 10, and sequenced on the Illumina MiSeq sequencer using an
273 indexed PE150 protocol with MiSeq V2 chemistry.

274

275 Paired-end reads were aligned to the reference genome [Genbank: CP002416] using
276 Burrows-Wheeler transform (Li and Durbin, 2009) and putative SNPs and small
277 indels were called using samtools and mpileup (Li et al., 2009). Putative structural
278 variants were called using a combination of BreakDancer (filtered to quality 90+)

279 (Chen et al., 2009), and Pindel (Chen et al., 2009). Variants occurring in less than
280 90 % of the reads and variants that were identical to those of the parent Δhpt strain
281 (i.e., due to errors in the reference sequence) were filtered out. Raw data is available
282 from the JGI Sequence Read Archive (JGI Project Id: 1064732).

283

284 *2.5. RNA isolation and ribosomal RNA removal*

285 Pelleted cells, 50 mL from each sample, were resuspended in 1 mL of TRIzol
286 (Invitrogen, Carlsbad, CA, USA) and lysed by bead beating with 0.8 g of 0.1 mm
287 glass beads (BioSpec Products, Bartlesville, OK, USA) for 3 x 20 seconds each at
288 6,500 rpm in a Precellys 24 high-throughput tissue homogenizer (Bertin
289 Technologies, Montigny-le-Bretonneux, France). The RNA from each cell lysate
290 was purified, DNaseI-treated, and quantity and quality assessed, as previously
291 described (Yang et al., 2012). High quality total RNA (RIN>8) was depleted of
292 rRNA using Ribo-Zero rRNA Removal Kit for bacteria (Epicentre-Illumina, San
293 Diego, USA) following the manufacturer's protocol. The depleted sample was
294 purified on a RNA Clean & Concentrator-5 (Zymo Research, Irvine, CA, USA)
295 following the manufacturer's protocol.

296 *2.6. Library preparation and sequencing*

297 Depleted RNA was used for RNA-Seq library preparation with the Epicentre
298 ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre-Illumina, San Diego, CA,
299 USA) following the manufacturer's protocol (EPILIT329 Rev.C). Agencount
300 AMPure beads (Beckman Coulter, Indianapolis, USA) were used to purify the
301 cDNA, and unique indexes were added during 13 cycles of library amplification.

302 The final RNA-Seq libraries were purified with Agencount AMPure beads
303 (Beckman Coulter, Indianapolis) and quantified with a Qubit fluorometer (Life
304 Technologies, Carlsbad, CA, USA). The library quality was assessed on a
305 Bioanalyzer DNA 7500 DNA Chip (Agilent, Santa Clara, CA, USA), and samples
306 were pooled and diluted. Sequencing was completed using a SR50 sequencing
307 protocol and V4 chemistry on an Illumina HiSeq 2500 platform (HudsonAlpha
308 Genomic Services Laboratory; Huntsville, AL).

309

310 *2.7. RNA-Seq analysis*

311 Raw reads were mapped to genome [GenBank:CP002416] using CLC Genomics
312 Workbench version 8.0 (CLC bio, Aarhus, Denmark) and the default settings for
313 prokaryote genomes. Counts of uniquely mapped reads were analyzed for
314 differential gene expression by DESeq2 (Love et al., 2014). Filtering was applied to
315 identify those genes with an FDR <0.05 and a greater than a $\log_2$ of +/-1 for
316 differential gene expression. Raw RNA-Seq data have been deposited in NCBI
317 Sequence Read Archive under accession SRP070709 and gene expression data
318 under NCBI GEO accession GSE78219.

319

320 *2.8. Metabolite extraction and sample preparation for quantification*

321 *C. thermocellum* intracellular metabolite extraction was performed anaerobically in
322 a Coy anaerobic chamber (Grass Lake, MI). Eight milliliters of culture (removed
323 with a 10 ml syringe) was deposited by vacuum filtration onto a 0.2 μ m nylon
324 membrane (47 mm diameter). The membrane was then placed (cells down) into 1.5

325 ml cold (-20°C or on dry ice) extraction solvent (20:20:10 v/v/v acetonitrile,
326 methanol, water) in a small petri disk and swirled. After a few moments the filter
327 was inverted (cells up) and solvent was passed over the surface of the membrane
328 several times to maximize extraction. Finally, the cell extract was stored at -80°C.
329 To measure extracellular metabolites, 2 ml of media was collected by filtration
330 using a 0.22 um syringe filter and stored at -80°C.

331

332 Cell extracts from *E. coli* (K-12 substrain MG1655 *rph*⁺ *ilvG*⁺) grown in ¹³C-
333 glucose were used as internal standards for quantitation of metabolites in *C.*
334 *thermocellum* strains. *E. coli* cells were grown aerobically to an OD₆₀₀ of ~0.45 in
335 M9 minimal medium containing 0.4% universally labeled ¹³C-glucose as sole
336 carbon source. To assure complete labeling of metabolites in *E. coli*, inoculation
337 was performed using 1/50 dilution from an overnight culture that was also grown on
338 ¹³C-labeled glucose. *E. coli* metabolites were extracted in a similar manner to the
339 procedure used for *C. thermocellum* (above), but under aerobic (benchtop)
340 conditions.

341

342 Extracellular metabolites were measured by empirically determining that
343 approximately 229 µl of media was retained on the nylon membrane during the
344 metabolite extraction process. Therefore, this amount of filtered medium (from the 2
345 ml collected as above) was thawed and mixed with 1.5 ml of extraction solvent. This
346 was then processed in parallel with the cellular samples as below. The volume of

extracellular medium was found to be variable (probably dependent on co-deposited cell volume) and so the exact amount was calculated (described below).

C. thermocellum and ^{13}C -labeled *E. coli* extracts were thawed, centrifuged at 14,000 RPMs for 10 min to remove cell debris and the *C. thermocellum* supernatants mixed with ^{13}C -labeled *E. coli* supernatant in ratios of 1:5, 1:1, 5:1 and 1:0 (no ^{13}C labeled internal standards). These mixed samples were then fully dried under a stream of nitrogen and subsequently re-suspended with solvent A (below) in half the initial volume. Samples were then centrifuged to remove particulates, transferred to HPLC vials, and analyzed by HPLC/MS.

Intracellular metabolite concentration for those metabolites with significant extracellular presence were estimated by subtracting the extracellular from intracellular values. To ensure accuracy glucose was used as an internal standard to determine the exact amount of extracellular medium volume co-deposited with the cells. This factor was then used to adjust the estimated concentration of extracellular metabolites.

2.9 Metabolite quantification

Samples were analyzed using an HPLC-MS/MS system consisting of a Dionex UHPLC coupled by electrospray ionization (ESI; negative mode) to a hybrid quadrupole - high-resolution mass spectrometer (Q Exactive orbitrap, Thermo

Scientific) operated in full scan mode for detection of targeted compounds based on their accurate masses and retention times (matched to purified standards). Liquid chromatography (LC) separation was achieved using an ACQUITY UPLC® BEH C18 (2.1 x 100 mm column, 1.7µm particle size). Solvent A was 97:3 water:methanol with 10 mM tributylamine (TBA) and approximately 9.8 mM acetic acid, pH ~8.2; solvent B was 100% methanol. Total run time was 24.5 min with the following gradient: 0 minutes, 5% B; 2.5 minutes, ramp from 5% B to 95% B over 14.5 minutes; hold at 95% B for 2.5 minutes; return to 5% B over 0.5 minutes; hold at 5% B for 5 minutes. All samples were injected twice (analytical replicates). MS scans consisted of full MS scanning for m/z between 70-1000 from time 0 to 18.5 minutes. Exceptions were: m/z of 207-210 excluded between 2-3 min.; and m/z 190-192 excluded between 11-12 minutes. Metabolite peaks were identified using Metabolomics Analysis and Visualization Engine (MAVEN) (Clasquin et al., 2012; Melamud et al., 2010).

382

383 *2.10. Calculations*

CO₂ produced was calculated based on the expected equimolar ratio of C₁:C₂ compounds plus the additional CO₂ liberated via valine biosynthesis, whereby CO₂ = [(ethanol + acetate + valine)-(formate)] Ratios of oxidized to reduced fermentation products (O/R) were calculated using reduction values of each fermentation product, calculated as the number of oxygen atoms less one-half the number of hydrogens in each compound (Moat et al., 2002). Carbon bound electron

equivalents were calculated as described by Harris *et al.* (Harris and Adams, 1979) and were used to determine electron recovery.

3. Results

3.1. Genomic analysis of nitrogen assimilation

The genome of *C. thermocellum* encodes one NADPH-dependent glutamate dehydrogenase (*gdh*; Clo1313_1847), one cluster of glutamine-oxoglutarate aminotransferase/glutamate synthase type enzymes (*gogat*; Clo1313_2032-2036), and four glutamine synthetases, including three Type III glutamine synthetases (*glnN*; Clo1313_1357, 2038, 2303) approximately 700 amino acids in length, and one 440 amino acid Type I glutamine synthetase (*glnA*; Clo1313_2031) (Figure 1). Biocyc (Karp, 2005; Romero and Karp, 2004) and DOOR (Dam et al., 2007; Mao et al., 2009) predict Clo1313_2030-2036 to be a single operon, and the electron carrier is unknown. Organization of genes and predicted operons involved in nitrogen assimilation are provided in Supplementary File 4. In principle, any two of these three enzymatic reactions (GLDH, GOGAT, and GS) should be sufficient to assimilate NH_4^+ , but the energetic cost differs depending on whether the ATP-hydrolyzing GS is utilized. Clo1313_2030 is predicted to encode an ANTAR domain protein/response regulator receiver (*nitR*) that is associated with regulation of nitrogen metabolism. Ammonium is likely transported by Clo1313_2260, which is an apparent fusion protein of an ammonium transporter (AmtB) with the regulatory protein GlnK (also called P_{II}). Urea is known to be a nitrogen source for *C. thermocellum*, and it encodes a urease gene cluster, including genes encoding the

413 urease enzyme (Clo1313_2490-2492), urease maturation genes (Clo1313_2486-
 414 2488), and a urea transporter (Clo1313_2493-2497). *C. thermocellum* also encodes
 415 putative nitrogenase subunits *nifD*, *nifK*, and *nifH* (Clo1313_2331, 2332, and 2339,
 416 respectively); however, nitrogenase activity has not been detected and N₂ does not
 417 seem to serve as a nitrogen source (Bogdahn and Kleiner, 1986; Kridelbaugh et al.,
 418 2013).

419

420

421 **Figure 1: Nitrogen assimilation pathway in *C. thermocellum* based on genome**
 422 **annotation.** *glnA*, Type I glutamine synthetase; *glnN*, Type III glutamine
 423 synthetase; *gogat*, glutamine-oxoglutarate aminotransferase/glutamate synthase type
 424 enzymes; *gldh*, glutamate dehydrogenase. Dashed lines indicate multiple enzymatic
 425 steps.

426

427 3.2. Deletion of Type I glutamine synthetase decreases amino acid secretion and
428 increases ethanol production

429 Glutamine synthetases allow for high efficiency NH_4^+ uptake by coupling
430 assimilation to ATP hydrolysis, providing a thermodynamic driving force. GlnA is
431 the most highly expressed glutamine synthetase in *C. thermocellum* (Gowen and
432 Fong, 2010; Wilson et al., 2013), and it is also the only Type I enzyme, which can
433 have both catalytic and regulatory functions. Therefore, the *glnA* gene,
434 Clo1313_2031, was deleted in *C. thermocellum* Δhpt , resulting in strain $\Delta hpt\Delta glnA$,
435 which will be referred to as $\Delta glnA$. *C. thermocellum* Δhpt (Argyros et al., 2011)
436 was used as the parent strain given that its growth and product profiles were the
437 same as the wild type DSM1313 strain and allowed for a selection method for our
438 deletion process. *C. thermocellum* Δhpt and $\Delta glnA$ were grown on 4.5 g/l (13.1
439 mM) cellobiose in defined medium (MTC₅). Deletion of *glnA* had no impact on
440 growth, as both Δhpt and $\Delta glnA$ strains grew to a final OD₆₀₀ of ~0.88 and had a
441 mid-exponential phase generation time of 1.3 h (± 0.1) (Supplementary File 5a).
442 Total elemental carbon in cell pellets for Δhpt and $\Delta glnA$ was 0.252 (± 0.010) and
443 0.269 g/l (± 0.017), respectively (corresponding to 21.0 and 22.4 mM of carbon,
444 respectively), and total pellet nitrogen was 0.062 (± 0.001) and 0.069 (0.002) g/l,
445 respectively, further demonstrating that deletion of *glnA* had no significant impact
446 on microbial biomass production. Interestingly, the initial pH of MTC₅ (pH = $7.43 \pm$
447 0.06) decreased to only 6.64 ± 0.13 in $\Delta glnA$ cultures versus 5.86 ± 0.16 in Δhpt
448 cultures.

449

450 Fermentation products were measured following near-completion of cellobiose
451 utilization (<1.0 mM remaining), approximately 10 hours after peak OD was
452 reached. Deletion of *glnA* increased ethanol production by 53%, improving ethanol
453 yields from 0.86 to 1.22 moles per mole hexose equivalent (Figure 2a, Table 1,
454 Supplementary File 5b). While negligible changes in formate, acetate, and
455 extracellular pyruvate were observed, lactate production was nearly completely
456 abolished and H₂ production decreased by 38%. Furthermore, total amino acid
457 secretion decreased by 44%, with the most abundant secreted amino acids, valine
458 and alanine, decreasing by 53% and 46%, respectively (Figure 2b). Conversely,
459 carbon and electron recoveries from canonical fermentation products and biomass
460 increased by 9.5% and 8.6%, respectively, in $\Delta glnA$ fermentations (Table 1),
461 demonstrating that additional carbon and electron flux is diverted from secreted
462 amino acids to canonical fermentation products. While total carbon and electron
463 recoveries were both c.a. 100% in $\Delta glnA$ fermentations, recoveries were 4.3% and
464 4.9% lower, respectively, in Δhpt fermentations, suggesting that some products may
465 be unaccounted for in the latter.

466 **A)**

467

468 **B)**

469

470 **Figure 2. Final fermentation products (A) and secreted amino acids (B) of *C.***
 471 ***thermocellum* strains.** All products were measured upon completion of cellobiose
 472 utilization. Grey bars, Δhpt ; black bars, $\Delta glnA$; (Cys)₂, cystine. Only amino acids
 473 with a change of greater than 0.08 mM compared to MTC₅ medium are shown.
 474 Only total secreted amino acid values are presented in Figure 2A. Negative values
 475 in Figure 2B indicate consumption of amino acids from the growth medium.

476

477 **Table 1. Final product yields and fermentation balances of Δhpt and $\Delta glnA$ on 4.5 g/l cellobiose in batch cultures**
 478 **grown in MTC₅.** Carbon recoveries of canonical fermentation products including biomass carbon (FPs), secreted amino
 479 acids (AAs), and total secreted products are provided.

	Product Yields (mol mol-hexose consumed ⁻¹)							C recovery (%) ²			e ⁻ recovery (%) ²		
	Ethanol	H ₂	Formate	Acetate	Lactate	CO ₂ ¹	Biomass carbon	FPs	AAs	Total	FPs	AAs	Total
CTFUD													
Δhpt	0.86	0.50	0.29	0.30	0.11	0.96	0.85	85.8	8.9	94.6	86.0	10.3	96.4
$\Delta glnA$	1.22	0.29	0.24	0.28	0.01	1.31	0.85	96.3	4.6	99.9	95.3	5.4	100.1

480

481 ¹CO₂ was calculated using C1:C2 ratio using ethanol, acetate, valine and formate concentrations.

482 ²Carbon and electron recoveries were calculated based on substrate (cellobiose), canonical end-product (ethanol, H₂,
 483 formate, acetate, lactate, pyruvate, glucose), secreted amino acid, and elemental carbon biomass measurements, and
 484 calculated CO₂ (from C₁:C₂ ratio).

485 3.3. *Unintended mutations are unlikely to be responsible for high-ethanol yielding*
486 *phenotype*

487 During strain construction, unintended mutations can be fixed in the population due
488 to the single colony purification steps. While most of these mutations do not result
489 in a noticeable phenotype, it was possible that unintended mutations were
490 responsible for increased ethanol production in $\Delta glnA$. Therefore, the mutant strain
491 was resequenced and compared to the parent strain. A total of 7 high-confidence
492 mutations were found in $\Delta glnA$ (Table 2). As expected, resequencing analysis
493 identified the deletion of *glnA*. Three genes in $\Delta glnA$ had non-synonymous
494 mutations. These included genes encoding (i) a family 18 glycoside hydrolase
495 predicted by KOG and CAZy to be a chitinase, (ii) a protein of unknown function,
496 and (iii) a hemerythrin-like metal-binding protein found upstream of chemotaxis
497 protein CheC. One 35 bp deletion was identified upstream and within the coding
498 region of a putative methyl-accepting chemotaxis sensory transducer, eliminating
499 the ribosome binding site and ATG start codon. Two other mutations were found in
500 intergenic regions (Table 2) that could have altered expression of the adjacent genes.
501 RNAseq analysis (see below) revealed that only expression of the hypothetical
502 protein Clo1313_1767 was affected (increased 4-fold).

Table 2. High confidence mutations found in $\Delta glnA$ that are not found in the parent Δhpt strain. INS, insertion; DEL, deletion. Changes in amino acids within coding region mutations are indicated

Position		Strand	Region	Locus tags	Genes	Type
Start	Stop					
2395922	2397259	-	CODING	<u>Clo1313_2031</u>	<u>glutamine synthetase, type I (<i>glnA</i>)</u>	DEL
491357	491357	+	CODING	Clo1313_0436	glycoside hydrolase family 18	W258R
2327020	2327020	-	CODING	Clo1313_1979	protein of unknown function UPF0027	D360E
3494201	3494201	+	CODING	Clo1313_2980	hemerythrin-like metal-binding protein	V45E
2131552	2131586	-	CODING/ INTERGENIC	Clo1313_1820	methyl-accepting chemotaxis sensory transducer	DEL
1728952	1728952	+	INTERGENIC	Clo1313_1476 (up)	Peptidase S7 flavivirus helicase (NS3)	INS
				Clo1313_1477 (down)	glycoside hydrolase family 9	
2060557	2060557	-	INTERGENIC	Clo1313_1767 (up)	Hypothetical protein	INS
				Clo1313_1768 (down)	cellulosome anchoring protein cohesin region	

507
508 To rule out the possibility that these mutations are the cause of the high-ethanol
509 yielding phenotype observed in $\Delta glnA$, we reconstructed the deletion again from the
510 parent strain as detailed in the Materials and Methods. We again observed a 42%
511 increase in ethanol production compared to the Δhpt , elimination of lactate
512 production, and a decrease in H₂ production (Supplementary file 5b), further
513 solidifying that deletion of *glnA* is responsible for the substantial increase in ethanol
514 production.

515

516 3.4. Expression of genes involved in nitrogen metabolism increased in $\Delta glnA$

517 To elucidate the mechanism of increased ethanol production and decreased amino
518 acid secretion in $\Delta glnA$, we performed RNA-seq analysis of $\Delta glnA$ and the parent
519 strain on mid-exponential phase batch cultures (OD₆₀₀ ~ 0.35). On average, 18.4
520 and 22.8 million reads were generated for each of the four Δhpt and $\Delta glnA$
521 replicates, respectively. 3009 and 3031 genes were identified with a minimum
522 average of two reads per gene for Δhpt and $\Delta glnA$, respectively (Supplementary File
523 6). Of these genes, 151 were shown to be differentially expressed by a factor >2 and
524 a p-value <0.05.

525

526 While Type I glutamine synthetase was the most highly expressed glutamine
527 synthetase (Rank 532; 5264 average reads) in Δhpt , Clo1313_1357 encoding one of
528 the three Type III glutamine synthetases was also highly expressed (Rank 634; 4410
529 average reads). The two other Type III glutamine synthetases (Clo1313_2038 and

530 Clo1313_2303) were expressed at low levels (1256 and 25 average reads,
531 respectively) in Δhpt . However, in $\Delta glnA$, expression of Clo1313_2038 and
532 Clo1313_2303 increased by 13 and 77-fold, respectively. The putative pseudogene
533 Clo1313_2037, annotated as an aspartate ammonia ligase and located upstream of
534 Clo1313_2038, was also upregulated 9.7-fold. While the GOGAT encoding genes
535 (Clo1313_2030-2036) are predicted to be an operon by DOOR and Biocyc, three of
536 these subunits (Clo1313_2034-2036) increased by 19-40-fold in $\Delta glnA$, while
537 subunits Clo1313_2032-2033 and Clo1313_2030 increased only 1.7- to 1.9-fold.
538 Notably, Clo1313_2034-2036 were expressed at much lower levels (~17-449
539 average reads) when compared to those of the other genes in the predicted operon
540 (2623-7082 average reads) in Δhpt . No significant change in expression was
541 observed for glutamate dehydrogenase (Clo1313_1847).

542

543 Deletion of *glnA* also caused a strong increase in expression of genes involved in
544 urea uptake and metabolism. The Clo1313_2486-2499 gene cluster, encoding a
545 multi-sensor hybrid histidine kinase and a two component AraC family
546 transcriptional regulator (Clo1313_2499 and Clo1313_2498, respectively), a urea
547 binding protein (Clo1313_2497), the urea ABC transporter (Clo1313_2493-2496),
548 and urease (Clo1313_2490-2492) and urease accessory protein (Clo1313_2486-
549 2488), were upregulated 6.7 to 81-fold in $\Delta glnA$. Additionally, the ammonium
550 transporter-P_{II} fusion (Clo1313_2260) was also upregulated by 35-fold in the
551 deletion strain.

552

Two additional gene clusters were also upregulated. First, genes encoding an RNF-complex (Clo1313_0061-0066) involved in electron transfer reactions between ferredoxin and NADH were upregulated 1.6 to 2.9-fold. Similarly, a gene cluster (Clo1313_0073-0080) encoding a putative glycerol kinase (Clo1313_0073), transketolase (Clo1313_0074-0075), alcohol dehydrogenase GroES domain protein (Clo1313_0076), ABC transporter (Clo1313_0077-0079), and phosphoglycerate mutase (Clo1313_0080) was also upregulated 2.4 to 3.4-fold. An increase was also observed in the transcription of some genes involved in protein synthesis, sporulation/germination, and a proton-translocating pyrophosphatase (Supplementary File 6).

563

While a number of genes were down-regulated, the decrease was less pronounced than the increase in gene clusters encoding genes involved in urea metabolism and nitrogen assimilation. Expression of the most highly down-regulated gene (Clo1313_2176), encoding a 4626 amino acid protein with an S-layer homology domain (SLH), decreased 4.3-fold in $\Delta glnA$. Other down-regulated genes include putative copper amine oxidases, genes predicted to be involved in carbohydrate hydrolysis, putative fatty acid metabolism genes, and Type II excretion system proteins predicted to be involved in pilus assembly (Supplementary File 6).

572

3.5. Intracellular glutamine levels are higher in $\Delta glnA$

Metabolomic analysis was performed to evaluate changes in metabolite pools in $\Delta glnA$. A total of 53 metabolites were identified, including nine in which

extracellular concentrations prevented accurate quantification of intracellular levels (Supplementary file 7). In these cases, changes in extracellular levels are used as a proxy for intracellular changes (Granucci et al., 2015; Paczia et al., 2012; Reaves et al., 2013). Fourteen metabolites had significant changes relative to the parent strain. Notably, glutamine levels increased 6.4-fold and extracellular α -ketoglutarate levels increased 5.5-fold in $\Delta glnA$, while glutamate levels remained the same. Threonine and dihydroxyoorotate levels increased 7.2 and 4.9-fold, respectively. While NADP⁺ levels increased 2.6-fold, NADPH, NAD⁺, and NADH pools did not change. GDP and GTP levels increased 2.9 and 1.8-fold respectively. Ornithine, the only metabolite measured in the urea cycle, also increased 1.8-fold. Conversely, the only intracellular metabolite with decreased levels in the mutant strain was acetyl-CoA levels, which decreased 2.5-fold.

588

589 **4. Discussion**

Metabolic engineering strategies aimed at simultaneously eliminating all canonical organic fermentation products, including lactate, acetate, formate, and decreased H₂ through elimination of an [FeFe] hydrogenase maturation factor, have improved *C. thermocellum* ethanol yields to ~70% of theoretical (Papanek et al., 2015). However, additional strategies are needed to redirect flux away from non-traditional fermentation products such as amino acids towards ethanol. The deletion of the Type I glutamine synthetase in an essentially wild type genetic background described here not only reduced secreted amino acids by 44%, but also improved ethanol production by 53%, suggesting that perturbation of nitrogen metabolism

599 could be an attractive approach for the further engineering of advanced *C.*
600 *thermocellum* strains. Remarkably, disruption of this nitrogen assimilation gene had
601 a more significant improvement on ethanol yields than independent deletions of *ldh*
602 (Argyros et al., 2011; Biswas et al., 2014), *pta* (Tripathi et al., 2010) or *pfl* (Rydzak
603 et al., 2015).

604

605 While redirecting carbon and electron flux away from secreted amino acids towards
606 canonical fermentation products could in part explain increased ethanol yields, the
607 increase in ethanol production (approximately 10 mM) far exceeds the decrease in
608 amino acid secretion (approximately 1 mM), suggesting additional mechanisms for
609 improved ethanol yields. The increase in ethanol can potentially be explained by
610 increased NADH availability for ethanol production due to a combination of
611 increased flux through Rnf and GOGAT (Figure 3). In *C. thermocellum*, catabolism
612 of pyruvate to acetyl-CoA via pyruvate:ferredoxin oxidoreductase (PFOR) generates
613 reduced ferredoxin (Rydzak et al., 2014; Rydzak et al., 2009; Rydzak et al., 2012),
614 which is not a direct electron donor for ethanol production. This reduced ferredoxin
615 is typically reoxidized through either (i) H₂ formation via Ech or bifurcating
616 hydrogenase, (ii) NADPH formation via NADH-dependent reduced
617 ferredoxin:NADP⁺ oxidoreductase (NfnAB) (Rydzak et al., 2014; Wang et al.,
618 2010), or (iii) NADH formation using Rnf. Given the decrease in H₂ production,
619 decrease in NADPH:NADP⁺ ratios, and increase in Rnf expression in $\Delta glnA$, we
620 hypothesize that Rnf is transferring electrons from ferredoxin to produce NADH,
621 which in turn can be directly used to reduce acetyl-CoA into ethanol. While steady

622 state ratios of NADH:NAD⁺ did not change in $\Delta glnA$, levels of intracellular acetyl-
623 CoA decreased 2.5-fold, suggesting that increased electron flux to NADH from Fd
624 may accelerate acetyl-CoA reduction into ethanol.

625

626 In addition to increased ethanol production, deletion of the only annotated Type I
627 glutamine synthetase (*glnA*) appears to have resulted in deregulation of nitrogen
628 metabolism. Despite the presence of excess ammonium (28 mM) and urea (33.3
629 mM) in the medium, genes encoding urea metabolism and transport, ammonium
630 transport, and ammonium assimilation, including GOGAT and two of the three
631 putative Type III glutamine synthetases, were highly upregulated. The upregulation
632 of urea metabolism genes presumably increased the rate of urea breakdown to
633 ammonia (and CO₂), resulting in the higher final pH of $\Delta glnA$ when compared to
634 Δhpt cultures. Furthermore, the upregulation of GOGAT may be responsible for the
635 observed increase of intracellular glutamine and α -ketoglutarate levels in $\Delta glnA$,
636 though the cofactor specificity and *in vivo* directionality of GOGAT are unknown.

637 Most transamination reactions involved in amino acid synthesis use glutamate as the
638 amino donor, rather than glutamine (Reitzer, 2003), producing α -ketoglutarate.

639 Because α -ketoglutarate levels were 5-fold higher while intracellular glutamate
640 levels did not change in $\Delta glnA$, the thermodynamics of transamination would be less
641 favorable, possibly resulting in decreased rates of amino acid production.

642 Furthermore, the decrease in the NADPH to NADP⁺ ratio in $\Delta glnA$ may also
643 decrease the free energy of NADPH-dependent anabolic amino acid reactions.

644 Together, these changes likely account for the decrease in amino acid production.

Figure 3: Proposed flux distribution in $\Delta glnA$ based on product profiles, transcriptomics, and metabolomic analysis. Ratios ($\Delta glnA/\Delta hpt$) of product yields, transcription, and metabolite levels are provided. Black solid arrows depict a proposed increase flux while grey dotted arrows depict a proposed decrease in flux in $\Delta glnA$ when compared to Δhpt .

646
647 Similar changes in expression of genes involved in nitrogen metabolism have been
648 observed in response to ethanol stress in *C. thermocellum* ATCC 27405 (Yang et al.,
649 2012). Specifically, genes encoding urea transport and metabolism, two Type III
650 glutamine synthetases (Clo1313_2038, 2303), and the first 3 subunits of GOGAT
651 (Clo1313_2034-2036) were up-regulated in response to ethanol stress. However,
652 Type I glutamine synthetase and ammonium transporter-P_{II} fusion were not
653 differentially expressed. Given that ethanol production increased in $\Delta glnA$, it could
654 be possible that increased ethanol concentrations may have contributed to the
655 changes in expression of nitrogen metabolism genes, but the concentration of added
656 ethanol in the previous study was substantially higher than the amount produced
657 here. The *hydG* deletion strain also has similarly high ethanol yields to $\Delta glnA$
658 (Biswas et al., 2015) but does not show a similar transcriptomic response in
659 nitrogen-related genes (Biswas, unpublished). Furthermore, treatment of *C.*
660 *thermocellum* chemostat cultures with methyl viologen, which also improved
661 ethanol yields, resulted in a decrease in expression of genes encoding one of the
662 three Type III glutamine synthetases (Clo1313_2303) and GOGAT (Clo1313_2034-
663 2036) (Sander et al., 2015), suggesting that ethanol levels are not a major driver of
664 nitrogen assimilation genes expression.

665
666 With the exception of *C. saccharobutylicum* (Amon et al., 2010), the mechanisms
667 involved in controlling nitrogen assimilation in *C. thermocellum* and other
668 clostridia, are poorly understood. In *C. saccharobutylicum*, nitrogen assimilation is,

669 in part, regulated by the ANTAR-family RNA-binding anti-terminator protein NitR
 670 (Shu and Zhulin, 2002), which is activated by a yet unknown signal transducer
 671 under nitrogen limitation (Stutz et al., 2007), and allows read-through of terminator-
 672 like structures in the promoter regions of *glnA* (Woods and Reid, 1995) and
 673 glutamate synthase genes *gltAB* (Stutz et al., 2007). A unique RNA motif consisting
 674 of a pair of stem-loops acts as a general recognition element of ANTAR-based
 675 regulatory proteins, and has been shown through bioinformatic analysis to be
 676 present upstream of the ammonium transporter-GlnK (AmtB-GlnK; Clo1313_2260)
 677 in *C. thermocellum* (Ramesh et al., 2012). Thus, up-regulation of NitR in *C.*
 678 *thermocellum* Δ *glnA* may increase expression of AmtB-GlnK and other proteins
 679 involved in nitrogen metabolism. Furthermore, in characterized organisms, GlnK
 680 senses intracellular α -ketoglutarate levels that are typically elevated under nitrogen
 681 starvation (Dodsworth et al., 2005; Radchenko et al., 2010; Yuan et al., 2009) and
 682 controls a wide range of processes involved in nitrogen metabolism through protein-
 683 protein interactions (Arcondeguy et al., 2001; Forchhammer, 2008; Huergo et al.,
 684 2013; Sarkar et al., 2012). The increased expression of NitR and GlnK and
 685 increased levels of α -ketoglutarate likely account for the observed changes in
 686 mRNA expression in the mutant strain.
 687
 688 In addition to α -ketoglutarate, other effector molecules also regulate nitrogen
 689 metabolism through gene expression and/or protein activity, including ammonium in
 690 Actinobacteria such as *Corynebacterium glutamicum* (Muller et al., 2006; Nolden et
 691 al., 2001; Rehm and Burkovski, 2011; Rehm et al., 2010) and glutamine in

692 Proteobacteria (Ikeda et al., 1996; Jiang et al., 1998; Reitzer, 2003) and Firmicutes
693 such as *Bacillus subtilis* (Wray et al., 2001). While high α -ketoglutarate levels in
694 the latter organisms indicate nitrogen starvation, high glutamine and low α -
695 ketoglutarate levels are typically indicative of nitrogen-rich conditions. In *B.*
696 *subtilis*, glutamine inhibits activity of glutamine synthetase (Deuel and Prusiner,
697 1974), and this feedback-inhibited form of glutamine synthetase can directly interact
698 with the global transcriptional regulator TnrA, preventing it from both binding DNA
699 and activating expression of genes involved in ammonium transport, urea
700 metabolism, and ammonium assimilation under nitrogen rich conditions (Wray et
701 al., 2001). While no TnrA homologue was identified in *C. thermocellum*, it is
702 feasible that another regulatory protein in *C. thermocellum* acts as a transcriptional
703 regulator of nitrogen metabolism genes and similarly responds to feedback-inhibited
704 glutamine synthetase. Together, the high levels of both glutamine and α -
705 ketoglutarate in *C. thermocellum* $\Delta glnA$ seemingly signals nitrogen-rich and -poor
706 conditions simultaneously, resulting in a transcriptional response that is indicative of
707 starvation and a flux response that suggests nitrogen excess.

708

709

710 **5. Conclusion**

711 While previous genetic engineering strategies were successful in improving ethanol
712 yields in *C. thermocellum* through elimination of canonical fermentation pathways,
713 ethanol yields were still too low, in part due to amino acid secretion. Deletion of the
714 Type I glutamine synthetase (*glnA*) not only reduced amino acid secretion, but also

715 increased ethanol production by 50%, demonstrating that genetic manipulation of
716 nitrogen assimilation pathways is a promising strategy for improving product yields
717 in *C. thermocellum* and potentially other organisms. Deletion of *glnA* increased
718 expression of nitrogen assimilation, ammonium transport, and urea uptake and
719 metabolism, despite the presence of excess nitrogen. These changes are typically
720 indicative of a nitrogen starvation response, but excess nitrogen was present during
721 growth, and that glutamine and α -ketoglutarate levels increased, suggesting that
722 nitrogen metabolism was deregulated in response to deletion of *glnA*. While the
723 regulatory mechanisms behind the changes in expression and product profiles in
724 $\Delta glnA$ remain to be elucidated, we propose that nitrogen metabolism is regulated by
725 NitR, GlnK, and GlnA itself, while the increase in ethanol is driven by increased
726 NADH availability via Rnf. Given that deletion of *glnA* resulted in a significant
727 increase in ethanol production, combining this deletion with other high-yielding *C.*
728 *thermocellum* strains may further improve ethanol yields.

729

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738

739

Figure 1: Nitrogen assimilation pathway in *C. thermocellum* based on genome annotation. *glnA*, Type I glutamine synthetase; *glnN*, Type III glutamine synthetase; *gogat*, glutamine-oxoglutarate aminotransferase/glutamate synthase type enzymes; *gldh*, glutamate dehydrogenase. Dashed lines indicate multiple enzyme steps.

Figure 2. Final fermentation products (A) and secreted amino acids (B) of *C. thermocellum* strains. All products were measured upon completion of cellobiose utilization. Grey bars, Δhpt ; black bars, $\Delta glnA$; (Cys)₂, cystine. Only amino acids with a change of greater than 0.08 mM compared to MTC₅ medium are shown. Only total secreted amino acid values are presented in Figure 2A. Negative values in Figure 2B indicate consumption of amino acids from the growth medium.

Figure 3: Proposed flux distribution in $\Delta glnA$ based on product profiles, transcriptomics, and metabolomic analysis. Ratios ($\Delta glnA/\Delta hpt$) of product yields, transcription, and metabolite levels are provided. Black solid arrows depict a proposed increase flux while grey dotted arrows depict a proposed decrease in flux in $\Delta glnA$ when compared to Δhpt .

Supplementary File 1: Primers used to construct and sequence deletion plasmid pNJ22::2031_del, and to verify deletion of *glnA* (Clo1313_2031) in Δhpt . Expected band sized in the parent strain, merodiploid, and deletion strain are provided.

Supplementary file 2: Key elements of pNJ22::2031_del used for deletion of Type I glutamine synthetases encoded by Clo1313_2031 (*glnA*). p15A ori, *E. coli* origin of replication; P-cbp, *C. thermocellum* cellobiose phosphorylase promoter; Teth39_2112 tdk, *Thermoanaerobacter pseudethanolicus* thymidine kinase; P-neo, neomycin phosphotransferase promoter; Neo (KanR), neomycin phosphotransferase conferring kanamycin resistance; pNW33N ori, *C. thermocellum* origin of replication; 2031 up, upstream sequence of homology to Clo1313_2031; 2031 down, downstream sequence of homology to Clo1313_2031; P-GapDH RBS, *C. thermocellum* glyceraldehyde-3-phosphate dehydrogenase promoter and ribosome binding site; Cat2, chloramphenicol acetyltransferase; Hpt2, hypoxanthine phosphoribosyltransferase; 2031 internal, internal sequence of homology to Clo1313_2031. Plasmid map (A) and Genbank file (B) of pNJ22::2031_del are provided.

Supplementary File 3: Deletion of Clo1313_2031 (*glnA*); overview and confirmation. A) Clo1313_2031 was deleted according to the protocol outlined by Argyros *et al.* (2011). Primer binding sites used to amplify the locus encompassing Clo1313_2031 (P1) and internal to Clo1313_2031 (P2 and P3) and corresponding expected product sizes in the parent and mutant strain are indicated. B) PCR confirmation of Clo1313_2031 deletion. Primer set P1 amplified the chromosomal

786 region that includes Clo1313_2031, and results in a 2668 bp fragment in the parent
787 strain and a 1399 bp fragment in Clo1313_2031 deletion strain. Primer sets P2 and
788 P3 amplified 331 bp and 485 bp fragments of the Clo1313_2031 gene, respectively,
789 in the Δhpt strain, but not in $\Delta glnA$. Reduction in size of the P3 amplicon and
790 absence of P1 and P2 amplicons confirms deletion of Clo1313_2031 in the mutant
791 strain.

792

793 **Supplementary File 4: Organization of genes and predicted operons involved**
794 **in nitrogen assimilation (green), ammonium transport (blue), urea metabolism**
795 **(purple), and putative regulation (orange).** Predicted operons are highlighted in
796 grey.

797

798 **Supplementary File 5: Summary of (A) growth data and (B) fermentation and**
799 **amino acid data for Δhpt , original $\Delta glnA$ isolate, and reconstituted $\Delta glnA$.**

800 Product yields and carbon and electron recoveries are provided.

801

802 **Supplementary File 6: Summary of RNAseq data.** Dataset contains total
803 RNAseq reads for each gene, the strain-specific rank of expression of each gene, the
804 mean of total normalized reads for the dataset (baseMean), standard error (lfcSE),
805 Wald statistic (stat), log2-fold changes in expression of genes in $\Delta glnA$ versus the
806 Δhpt parent strain, Wald test p-values, and BH adjusted p-values (padj).

807

808 **Supplementary File 7: Summary of metabolomic data.** Dataset contains all
809 intracellular and extracellular metabolites analyzed by GC-MS. In cases where
810 strong metabolite signals were found extracellularly, accurate measurements of
811 intracellular metabolites could not be achieved. Statistical significance of changes
812 in metabolite pools was assessed using the Students *t*-test.

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