Synthesis of heterologous mevalonic acid pathway enzymes in *Clostridium ljungdahlii* for the conversion of fructose and of syngas to mevalonate and isoprene

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Running Title: **Engineering *C. ljungdahlii* to produce isoprene**

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ABSTRACT

There is a growing interest in the use of microbial fermentation for the generation of high-demand, high-purity chemicals using cheap feedstocks, in an environmentally-friendly manner. One example explored here is the production of isoprene (C_5H_8), a hemiterpene, which is primarily polymerized to polyisoprene in synthetic rubber in tires, but which can also be converted to C10 and C15 biofuels.

The strictly anaerobic, acetogenic bacterium *Clostridium ljungdahlii*, used in all of the work described here, is capable of glycolysis using the Emben-Meyerhof-Parnas pathway and of carbon fixation using the Wood-Ljungdahl pathway. *Clostridium-E. coli* shuttle plasmids each bearing either 2 or 3 different heterologous genes of the eukaryotic mevalonic acid (MVA) pathway or eukaryotic isopentenyl pyrophosphate isomerase (Idi) and isoprene synthase (IspS) were constructed and electroporated into *C. ljungdahlii*. These plasmids, one or two different of which were introduced into the host cells, enabled the synthesis of mevalonate and of isoprene from fructose and from syngas (H_2, CO_2, CO) and the conversion of mevalonate to isoprene. All of the heterologous enzymes of the mevalonic acid (MVA) pathway as well as isopentenyl pyrophosphate isomerase (Idi) and isoprene synthase (IspS) were shown to be synthesized at high levels in *C. ljungdahlii*, as demonstrated by Western blotting, and were enzymatically active, as demonstrated by *in vivo* product synthesis.

The quantities of mevalonate and isoprene produced here are far below what would be required of a commercial production strain. Proposals are made, however, that could enable a substantial increase in the mass yield of product formation.

Importance:

This study demonstrates the ability to synthesize a heterologous metabolic pathway in *C. ljungdahlii*, an organism capable of metabolizing either simple sugars or syngas or both together (mixotrophy). Syngas is an inexpensive source of carbon and reducing equivalents and is produced as a major component of some industrial waste gas, and can be generated by gasification of cellulosic biowaste, and of municipal solid waste. Its conversion to useful products therefore offers potential cost and environmental benefits. The ability of *C. ljungdahlii* to grow mixotrophically also enables the recapture, should there be sufficient reducing equivalents available, of CO_2 released upon glycolysis, potentially increasing the mass yield of product formation. Isoprene is the simplest of the terpenoids and so demonstration of its production is a first step toward synthesis of higher-value products of the terpenoid pathway.

Keywords:

Clostridium ljungdahlii, acetogenic bacteria, Wood-Ljungdahl pathway, mevalonic acid pathway, syngas, fructose, metabolic engineering, isoprene, mevalonate, bioenergetics

INTRODUCTION

Isoprene is a hemiterpene (C_5H_8), used primarily for polymerization to cis-1,4-polyisoprene, the main constituent of natural rubber (as in vehicle tires, surgical gloves), and for styrene isoprene-styrene copolymer (as in thermoplastic rubber and adhesives). Isoprene can also be converted by oligomerization and hydrogenation to produce blendable saturated C10 and C15 biofuels (1). The annual global production of petrochemically-derived isoprene is close to one million tons (2). With the increase in the volatility of oil prices, with clean energy policies likely to result in a decline in U.S. petroleum consumption and thus in the availability of C_5 fractions

from naphtha cracking, and the significant threat of fungal disease to rubber plantations in Asia and Africa (3), there is an increased demand for a more sustainable and reliable generation of isoprene, particularly for the production of synthetic cis-1,4-polyisoprene for tires. Isoprene is also the simplest product of the terpenoid pathway. The ability to engineer the production of isoprene is therefore a first step toward the engineering of a host organism to produce this and higher-value terpenoids.

Two evolutionarily distinct pathways exist for the generation of isopentenyl-pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP), the starting point for all terpenoid biosynthesis, including isoprene. These are 1) the 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (DOXP/MEP) pathway, found in most prokaryotes and which begins with the condensation of pyruvate and glyceraldehyde-3-phosphate, and 2) the mevalonic acid (MVA) group of pathways found in most eukaryotes, archaea and some bacteria, which generally begins with the condensation of two acetyl-CoA. Efforts at aerobic microbial biosynthesis of isoprene from glucose using heterologous protein synthesis of the MVA pathway have shown higher rates, yields and titers of isoprene synthesis in *E. coli* compared with the native DOXP/MEP pathway (2, 4). The exact reason for the higher yields and titers with the MVA pathway is still unclear, but may have to do with reduced regulatory control associated with the expression of heterologous MVA pathway genes (e.g. from *Saccharomyces cerevisiae* and *Enterococcus faecalis*) or more severe flux bottlenecks in the DOXP/MEP pathway (4, 5).

In the approach demonstrated here, we use fructose and syngas individually as a source of carbon and reducing equivalents (6) to be metabolized to mevalonate and to isoprene in the acetogenic bacterium (7), *Clostridium ljungdahlii*. This organism is Gram-positive and a strict anaerobe, capable of autotrophic growth on H_2 + CO_2 or CO (syngas) or all three together using

92 the Wood-Ljungdahl (WL, reductive acetyl-CoA) pathway (8, 9), the final product of which is
93 acetyl-CoA. The conversion of syngas to isoprene via acetyl-CoA requires less energy using the
94 MVA pathway than it does for the DOXP/MEP pathway (which would require gluconeogenesis)
95 and so is the pathway of choice where ATP is limiting. *C. ljungdahlii* can also grow
96 heterotrophically on organic substrates (e.g. hexoses and pentoses (7, 10)) and mixotrophically,
97 refixing, via the WL pathway, the CO_2 liberated upon the decarboxylation of pyruvate to acetyl-
98 CoA in glycolysis (7, 11).

99 A genetic system has been developed for *C. ljungdahlii* (12, 13) and it has been demonstrated
100 to be capable of heterologous gene expression (12). In this paper, we will show that it is possible
101 to couple the Embden-Meyerhof-Parnas (EMP) and the Wood-Ljungdahl (WL) pathways to a
102 genetically-engineered eukaryotic MVA pathway in *C. ljungdahlii*, enabling the conversion of
103 fructose and of syngas to mevalonate and to isoprene. Syngas is likely to be a less expensive
104 feedstock than a hexose sugar on a mass basis as it can be generated by gasification (via steam or
105 oxygen reforming) of a wide range of inexpensive materials – e.g. natural gas, agricultural waste,
106 coal, and municipal solid waste (6). Low cost CO-enriched waste streams are also generated as a
107 consequence of industrial processes such as steel manufacture and oil refining, a source that has
108 been exploited by LanzaTech for the generation of biofuels and other biochemicals using
109 acetogenic microorganisms (6). That syngas can be derived from waste gas or from waste
110 materials means that there is potentially an environmental in addition to a cost benefit associated
111 with the use of such feedstocks.
112
113 **RESULTS**
114 **Plasmids used for the introduction of the MVA pathway genes**

115 Four different Gram-positive replicons are represented in the set of *Clostridium-E. coli*
116 shuttle plasmids used here (14) (see Materials and Methods). The most *C. ljungdahlii*
117 transformants were obtained with the pCB102 replicon (typically 50-100/µg plasmid DNA),
118 about one order of magnitude fewer with pBP1 and none with pCD6 and pIM13.
119
120 **Synthesis of heterologous MvaE and MvaS in pJF100-transformed *C. ljungdahlii***
121 Plasmid pJF100 was constructed (Table 1, Figure S3 and Supplementary File S2) with
122 the heterologous genes *mvaE* and *mvaS* (see Materials and Methods), inserted as an operon under
123 the control of the P*fdx* ferredoxin promoter, with the Cpa fdx ferredoxin terminator (from *C.*
124 *pasteurianum*). The plasmid was electroporated into *C. ljungdahlii* (see Materials and Methods
125 and Supplementary File S2).
126 *In vivo* synthesis of MvaE and MvaS in pJF100-transformed *C. ljungdahlii* grown on
127 MES-F medium (chamber gas atmosphere) was compared to that in *E. coli* TV3007 (Materials
128 and Methods), transformed with the same pJF100 plasmid (15). Western Blots (Materials and
129 Methods, Figure S4 and Supplementary File S2) show high levels of both proteins. The parent
130 bands of MvaE and MvaS showed molecular masses consistent with the calculated 86.5 kDa and
131 42.1 kDa, respectively (15). The former, however, was partially proteolyzed, with two fragments
132 running between the 38 and 49 kDa markers.
133
134 **Synthesis of mevalonate in *C. ljungdahlii* grown on fructose**
135 *In vivo* enzymatic activity of MvaE and MvaS was demonstrated by the detection of
136 mevalonate in the growth medium. The WT *C. ljungdahlii*, and the pMTL83151 (Figure S3) and
137 pJF100 C. *ljungdahlii* transformants were grown to an OD_{600} of ~2 on MES-F medium in serum

bottles pierced to allow equilibration with the anaerobic chamber atmosphere. Aliquots of the culture medium were acidified to convert mevalonate to mevalonolactone (16), spun down and the supernatants injected into the HPLC (see Materials and Methods, Supplementary File S2).

The WT and the pMTL83151-transformed *C. ljungdahlii* showed no peak in the HPLC elution pattern (Figure 2) in the position of the mevalonolactone standard (19.3 min, RID signal). In contrast, the similarly-cultured pJF100 transformant showed a large mevalonolactone signal at this position. Correcting for dilution, there were ~0.3 mg/mL (2 mM) mevalonate in the culture medium for the pJF100 *C. ljungdahlii* transformant. Clearly, MvaE and MvaS in the pJF100 plasmid-transformed *C. ljungdahlii*, are active for the conversion of acetyl-CoA to mevalonate.

Synthesis of heterologous IspS and Idi in pJF100 Fdii-transformed *C. ljungdahlii*

The DOXP/MEP pathway, naturally present in *C. ljungdahlii* (17), generates IPP + DMAPP. *In vivo* synthesis of Idi and IspS should, therefore, enable the conversion of endogenous IPP + DMAPP to isoprene (Figure 1). Plasmid pJF100 Fdii was constructed (Table 1, Figure S5 and Supplementary File S2) by replacing the *mvaE* and *mvaS* genes of pJF100 with the heterologous genes, *idi* and *ispS* (Materials and Methods). Two preparations of the plasmid (#1 and #3) were transferred into *C. ljungdahlii*.

Western blots of the *C. ljungdahlii* pJF100 Fdii transformants and of *E. coli* TV3007, transformed with related plasmid, pJF200 (Figures S5 and S6 and Supplementary File S2), show high levels of both proteins. The parent bands of IspS and Idi show molecular masses consistent with the calculated 62.6 kDa and 33.4 kDa, respectively (15), though the former was partially proteolyzed, generating two fragments of ~27-8 kDa.

Conversion of fructose to isoprene

In vivo enzymatic activity of the translated Idi and IspS was demonstrated by the detection of isoprene in the culture headspace. *C. ljungdahlii* WT and the pJF100 Fdii transformants (plasmids 1 and 3, see Supplementary File S2) were grown for one day in 10 mL of MES-F medium in 20 mL Agilent vials at 37°. The vial headspace was sampled and analyzed by GC/MS (Materials and Methods). Signals and spectra were compared to an isoprene standard.

The total ion mass spectra (Figures 3C and D) show a close match between the isoprene standard and the signal derived from the pJF100 Fdii (plasmid 1) transformant headspace, with mass spectrum m/z features of 68, 67, 53 and 39. Using single ion monitoring (Figure 3A and B, m/z=67), the *C. ljungdahlii* WT (black) and the pJF100 Fdii transformants (red and blue) all showed signals with the same retention time as the isoprene standard (1.73 min). The WT produced some isoprene, as do other Gram-positive and Gram-negative bacteria (18). The transformants, however, generated 4-5 times more isoprene (Table 2), indicating that Idi and IspS are enzymatically active in the pJF100 Fdii transformants. The reason for the slower growth of the transformant with plasmid 1 relative to plasmid 3 (see Figure 3 legend) is unclear and was not routinely observed. Both transformants, however, produced the same amount of isoprene.

***C. ljungdahlii* adaptation to growth on syngas and 6x His codon *ispS* modified pJF100 Fdii**

The *C. ljungdahlii* WT and transformants were adapted to growth on syngas (see Supplementary File S2) to demonstrate that the latter were capable of producing mevalonate and isoprene from syngas. However, adaptation of the pJF100 Fdii *C. ljungdahlii* transformant to growth on MES-0F (syngas atmosphere) required modification of the plasmid to produce N-terminal and C-terminal 6x His-tagged versions of IspS. Western blotting (Figure S7) detected

184 N- and C- terminally His-tagged IspS and Idi in the *C. ljungdahlii* pJF100 Fdii His-tagged IspS
185 transformants, indicating high levels of synthesis for each. These transformants produced
186 isoprene at levels similar to those shown by the pJF100 Fdii transformants when cultivated on
187 fructose (not shown). The reason for the difference in syngas adaptability of the pJF100 Fdii
188 transformants with and without the 6x His-tag on IspS is unclear. The doubling times of all of the
189 syngas-adapted strains were typically ≤24 h.
190
191 **Conversion of syngas to biomass and product**
192 To demonstrate that the syngas-adapted *C. ljungdahlii* pJF100 transformant was indeed
193 converting syngas to cell biomass and product, the cells were cultivated on MES-0F medium +
194 thiamphenicol at 30°C under either the syngas atmosphere or N_2 alone. Cultivation experiments
195 were performed in duplicate for 26 h in 4 mL medium in 16.5 mL Supelco vials. Under the
196 syngas atmosphere, the cell density increased from OD_{600} = 0.500 to 0.822 ±0.023, while under
197 the N_2 atmosphere, the cell density increased from OD_{600} = 0.500 to only 0.520 ±0. These results
198 indicate that cell growth requires the presence of syngas.
199 Multiple MES-0F-repassaged syngas cultures of the WT *C. ljungdahlii* and of the pJF100
200 and the pJF100 Fdii His-tagged IspS transformants were cultivated on MES-0F under the syngas
201 atmosphere for 26 or 20 h (see Table 3). The final cultures were started at 0.500 OD_{600}. Cell
202 density was followed at OD_{600} and the headspace was analyzed for the percent consumption of
203 CO (~50%) in that time and the mole ratio of CO_2 produced per CO consumed determined (see
204 Table 3). In all cases, the mole ratio of CO_2 produced/CO consumed was ≤0.44 indicating that
205 the syngas-adapted *C. ljungdahlii* pJF100 transformants were fixing CO and not just oxidizing it

to CO_2. These results show that CO was being converted to biomass and to any organic product generated (see below).

Production of mevalonate by *C. ljungdahlii* cultured on syngas

The production of mevalonate in the pJF100 *C. ljungdahlii* transformant cultured on syngas was confirmed using LC/MS (Figure 5, Materials and Methods). This strain was subjected to 2 passages, first in MES-0.01F and second in MES-0F (both under a syngas atmosphere), during which time the OD_{600} and the broth mevalonate concentrations were monitored (Figure 5). The mevalonate concentrations increased with cultivation time (Figure 5). Syngas-adapted WT *C. ljungdahlii*, grown on MES-0F (syngas atmosphere) for 7 d to an OD_{600} of 1.07. showed a concentration of mevalonate of 0.36 µg/mL, the same as that present in fresh MES-0F medium. Thus, the WT had produced no mevalonate, in contrast with the pJF100 transformant which had converted syngas to mevalonate.

Production of isoprene by *C. ljungdahlii* grown on syngas

The production of isoprene in the pJF100 Fdii N-term His tagged IspS transformant cultured on syngas was confirmed using solid phase microextraction (SPME) of the headspace coupled with GC/MS (see Materials and Methods). MES-0F-repassaged syngas cultures of the WT *C. ljungdahlii* and of the pJF100 Fdii N-term His-tagged IspS transformant were both started at 0.500 OD_{600} in 4 mL MES-0F (syngas atmosphere) in 16.5 mL Supelco vials and grown for 20 h at 30°C (final OD_{600}, 1.034 and 0.888, respectively, the same samples as in Table 3). The total and extracted-ion (EIC) chromatograms for the WT and the pJF100 Fdii N-term His-tagged IspS

228 transformant showed an elution peak at 10.25 min (Figure 4A, B, and C). The mass spectra in
229 each case showed a close fit with the reference isoprene spectrum (Figure 4D).

230 Table 2 shows the calculated isoprene concentrations detected by EIC at m/z=67 at a
231 retention time of 10.25 min. The average of two runs, the first using 20:1 split mode (Table 2)
232 and the second using splitless mode (Figure 4A, B, C and D) showed that the pJF100 Fdii N-
233 term His-tagged IspS strain produced 5.2±1.5 times more isoprene than the *C. ljungdahlii* WT.
234

235 **Synthesis of the lower mevalonate pathway enzymes + IspS + Idi**

236 It remains to be demonstrated that an active lower MVA pathway can be produced in *C.*
237 *ljungdahlii*. Plasmid pJF102 (Table 1, Figure S8, Supplementary Files S1 and S2) containing the
238 lower pathway genes, *mvk*, *pmk* and *mvd* (see Materials and Methods) was transferred to *C.*
239 *ljungdahlii* to enable production of Mvk, Pmk and Mvd (Figure 1), respectively. A second
240 plasmid, pJF101, subsequently transferred, (Table 1, Figure S8, Supplementary Files S1 and S2),
241 contains *idi* and *ispS*, enabling the conversion to isoprene by Idi and IspS of IPP and DMAPP,
242 produced by the lower MVA pathway. The plasmids contained two different clostridial
243 replicons, antibiotic resistance markers and promoters (see Table 1) to assure that both would
244 reside stably within the host cell. Their stepwise transfer into *C. ljungdahlii* is described in
245 Supplementary File S2.

246 Two of the doubly-transformed *C. ljungdahlii* strains (pJF101+pJF102 #4 and #5) were
247 grown on liquid MES-F medium containing both thiamphenicol and spectinomycin. Western
248 blotting (see Figure S9 and Supplementary File S2) showed that only #4 produced all 5
249 heterologous proteins.
250

Conversion of mevalonate to isoprene

In vivo conversion of exogenous mevalonate to isoprene was used as a test of enzyme activity. Fresh MES-F medium, containing 0, 10, 20 and 40 mM mevalonate and both antibiotics was inoculated at 0.4 OD_{600} with the pJF101+pJF102 #4 double transformant (37°C, 10 mL culture medium in 16.5 mL Supelco vials). After 15.5 h, only the vial containing no added mevalonate showed growth (OD_{600}=1.25). One mL of vial headspace was injected into the GC/MS. All three mevalonate-containing cultures produced isoprene (~140 ng per mL in the headspace, 61 ng isoprene per mL of broth), far higher than the amount of isoprene produced from fructose or syngas (Table 2). The culture with no added mevalonate produced ≤10% of this amount. Clearly, mevalonate was being converted to isoprene in the pJF101+pJF102 #4 double transformant, indicating that the synthesized heterologous proteins were all enzymatically active.

DISCUSSION

We have demonstrated that all of the heterologous enzymes of the eukaryotic MVA pathway plus Idi and IspS can be synthesized *in vivo* at high levels and in active form using *Clostridium-E. coli* shuttle plasmids in *C. ljungdahlii*. In transformants cultivated on fructose and on syngas, heterologous MvaE and MvaS (see Figure 1) catalyzed the production of mevalonate from acetyl-CoA, and heterologous Idi and IspS catalyzed the production of isoprene from IPP and DMAPP, produced via the endogenous DOXP/MEP pathway (17). Mvk, Pmk and Mvd, Idi and IspS (see Figure 1) together were able to catalyze *in vivo* the synthesis of isoprene from exogenous mevalonate.

The quantities of product (mevalonate and isoprene) produced here, while a proof of concept, are, however, far below what is required for commercial production. Increased isoprene

production is likely to require enhanced levels of heterologous enzyme synthesis, and further refinements directed at flux optimization, redox balance and ATP supply.

Comparison of cultivation conditions

The isoprene measurements give some indication of limitations in pathway flux. Table 2 indicates that considerably more isoprene (18 to 90-fold/cell) accumulates when generated from mevalonate using the lower MVA pathway plus Idi and IspS than when only the latter two enzymes are produced in fructose-grown cells. This difference likely results from a higher rate of synthesis and a higher intracellular concentration of IPP and DMAPP produced from mevalonate in the MVA pathway as opposed to that produced by the endogenous DOXP/MEP pathway (17) in cells grown on fructose or syngas. An accumulation of toxic phosphorylated intermediates derived from mevalonate, particularly IPP (5), is also a possible explanation for the inhibition of cell growth at ≥10 mM mevalonate. Growth inhibition by ≥10 mM mevalonate itself also remains a possibility. As shown earlier, pJF100-transformed *C. ljungdahlii* was able to produce 2 mM mevalonate while growing to an OD_{600} of 2 (Figure 2). Even the isoprene produced in the absence of added mevalonate was higher upon synthesis of the lower mevalonate pathway plus Idi and IspS than in the other cases described in Table 2. MES-F medium contains a very low concentration of mevalonate, 0.036 µg/mL or 2.4 µM (Figure 5), that is not inhibitory to cell growth and yet is in 20-fold molar excess relative to the amount of isoprene produced (~1.3 nmoles).

Considerably more mevalonate accumulates with fructose as the carbon source as opposed to syngas. With the cells growing to 2 OD in both cases, ~300 µg/mL accumulated in the presence of fructose as opposed to ≤68 µg/mL under syngas growth conditions. Growth on

297 syngas is also substantially slower (2-4 fold) than it is on fructose. This difference in metabolism
298 is likely a reflection of differences in the cellular energy charge in the two cases, 2-3 times
299 higher in fructose-grown as opposed to syngas-grown *C. ljungdahlii*, as determined in a
300 metabolomics study on *C. ljungdahlii* (Bruce Diner, Yifan Xu and Pat Mauvais, unpublished).
301 Glycolysis produces a net +2 ATP by substrate-level phosphorylation per 2 acetyl-CoA produced
302 in the EMP pathway. Net ATP production from syngas to acetyl-CoA is the result of the
303 operation of the Wood-Ljungdahl pathway (which consumes one ATP/acetyl-CoA) and the
304 chemiosmotic Rnf complex, coupled by the electrochemical membrane potential to the
305 cytoplasmic membrane-bound ATP synthase (19). Assuming that 3.66 H^+ are required per ATP
306 in the ATP synthase and assuming that all of the electron-bifurcating and Rnf proton pumping
307 mechanisms for energy conservation invoked by Mock *et al.* (20) (Figure 1) are operating in *C.*
308 *ljungdahlii* in association with the Wood-Ljungdahl pathway, one can calculate that -0.05 ATP
309 per acetyl-CoA are produced from H_2 + CO_2 and +0.50 ATP per acetyl-CoA are produced from
310 CO. The reason for the difference between H_2 and CO as the source of reducing equivalents is
311 because CO ($E^{0'}_{CO2/CO}$ = -520 mV) (21) is more reducing than is H_2 ($E^{0'}_{H+/H2}$ = -414 mV) (22,
312 23), increasing the amount of reduced ferredoxin that can be produced by CO and consequently
313 the amount of ATP that can be generated via the protonmotive force maintained by the Rnf
314 complex coupled to Fd_{red} + $NAD^+ \rightarrow Fd_{ox}$ + NADH (with a presumed $2H^+/2e^-$, see (19, 20)).
315 The 2 g yeast extract/L present in the MES media had little impact on carbon flux to
316 product in *C. ljungdahlii*. Syngas-adapted cells, cultivated on MES-0F under a N_2 atmosphere,
317 showed no sustained growth. We also found that >2x as many moles of CO were consumed as
318 CO_2 produced (Table 3) when *C. ljungdahlii* was grown on MES-0F and syngas, indicating that
319 a significant fraction of the carbon of CO was converted to biomass and product.

Bioenergetics considerations for syngas conversion to isoprene

A total of 6 ATP is required for the synthesis of isoprene from syngas, 3 for the synthesis of 3 acetyl-CoA via the Wood-Ljungdahl pathway and another 3 for the conversion via the MVA pathway of the 3 acetyl-CoA to isoprene. Bertsch and Müller (24) have argued that in *A. woodii* grown on syngas there is not sufficient ATP generated chemisomotically to enable the synthesis of isoprene. Some substrate-level ATP production via phosphotransacetylase and acetyl kinase from acetyl-CoA (Figure 1) is required. The electron bifurcating redox complexes differ in number and cofactor specificity in *C. ljungdahlii* relative to *A. woodii* (20, 24) and offer some bioenergetic advantage over the latter organism. However, our own calculations (not shown) indicate some shortfall in ATP generation in *C. ljungdahlii*, even if this organism were to use the same energy-conserving electron bifurcating complexes suggested to be operating in *C. autoethanogenum* (20) (Figure 1) including MTHFR, the presence of which remains speculative (25). As in the redox argument above, it is advantageous in the metabolic pathways to produce NADPH ($NADP^+$/NADPH (E'=-370mV, physiological conditions) (21) and to consume NADH (NAD^+/NADH, E'=-280mV, physiological conditions) (26) as NADPH (but not NADH) can generate Fd_{red} by Nfn electron bifurcation (Figure 1) (27). As an example, replacing the NADPH-dependent HMG-CoA reductase (MvaE) with one that is NADH dependent (28) adds +1 Fd_{red}. Assuming in addition 3.3 $H^+(Na^+)$/ATP (24) or 3.66 H^+/ATP (20) in the ATP synthase, then the ATP chemisosmotically generated using the Rnf complex is close to the 6 (we calculate 6.2 and 5.7, respectively) necessary for isoprene synthesis from CO. Allowing some substrate-level phosphorylation, derived from acetyl-CoA to acetate (Figure 1), would provide additional ATP, but at further cost to the mass yield of isoprene synthesis.

There are a number of options, yet to be explored, that might further enhance ATP synthesis. A H^+-dependent pyrophosphatase has been identified in prokaryotes (29-31) which if introduced into *C. ljungdahlii* and directed to the cytoplasmic membrane could contribute (2 H^+ translocated per PPi hydrolyzed (32)) to the membrane electrochemical proton potential. Doing so would require the use of internal sources of pyrophosphate (e.g. RNA, DNA, polysaccharide and fatty acyl-CoA synthesis) as an exogenous supply would likely require an expenditure of energy for PPi import.

Potential for further improvement in the energy balance

Coelho and coworkers (33) have proposed four anaerobic pathways, beginning with 2,3-dihydroxyisovvalerate (DHIV), for the synthesis of isoprene. DHIV can be generated from syngas and the WL pathway by acetyl-CoA→pyruvate→2-acetolactate→DHIV. While these pathways consume only 1 or 2 ATP as opposed to 3 for the MVA pathway conversion of acetyl-CoA to one isoprene, their realization would require the discovery of 5-7 new enzymes.

It was originally shown in *Clostridium aceticum* and *Moorella thermoacetica* that the 2 CO_2 produced per glucose in the EMP pathway could be refixed by the WL pathway using the eight reducing equivalents derived from glycolysis (7). There are sufficient reducing equivalents (24 e^-) and ATP (six) produced in glycolysis of 3 fructose to convert the 6 acetyl-CoA generated to 2 isoprene, via the MVA pathway. However, there is then no residual ATP and insufficient reducing equivalents to enable the 6 CO_2, generated at the same time, to be refixed and converted to isoprene.

There are potential solutions to both deficiencies. It was recently demonstrated in *C. ljungdahlii* that H_2 can provide additional reducing equivalents, enabling, through mixotrophic

366 metabolism, further CO_2 refixation in the production of acetone (11). A number of pathway
367 modifications of the MVA and glycolytic pathways could enable an enhancement in the
368 generation of ATP as well. These include, as above, in the MVA pathway, the replacement of the
369 NADPH-dependent HMG-CoA reductase with its NADH-dependent homologue and, in the
370 EMP pathway, replacement of the NAD-dependent with a NADP-dependent glyceraldehyde-3-
371 phosphate dehydrogenase (e.g. Z-P Guo *et al.*, 2011) (34) and the ATP-dependent
372 phosphofructokinase with its pyrophosphate-dependent homologue (e.g. from *Methylomonas*
373 *methanica* (35)) (see Figure 1). The combination of supplemental reducing equivalents, cofactor
374 replacement and three rather than two ATP per hexose sugar converted to acetyl-CoA via
375 glycolysis (36, 37) could enable, by CO_2 refixation, the conversion of 3 fructose to 3 isoprene.
376 Calculations (not shown) indicate that these changes in glycolytic substrate-level
377 phosphorylation plus chemiosmotic ATP synthesis could satisfy the overall ATP demand for
378 producing 3 isoprene from 3 fructose with a theoretical mass yield of 37%, an improvement over
379 the 25% yield of the classical EMP and MVA pathways.
380
381 **MATERIALS AND METHODS**
382 **Bacterial strains**
383 Wild-type (WT) *Clostridum ljungdahlii* (ATCC 55383) and transformants derived therefrom
384 were used for all of the experimental work in this paper. All strains were cultivated anaerobically
385 in MES-F, MES-0.1F, MES-0.01F or MES-0F (15), medium, containing 10 g/L, 1 g/L, 0.1g/L
386 and 0 g/L fructose, respectively, under an atmosphere of anaerobic chamber gas (2% H_2, 5%
387 CO_2, 93% N_2) or syngas (35% CO, 36% H_2, 18% CO_2 and 11% Ar). In MES-0F, syngas was the
388 principal source of carbon and reducing equivalents. Growth of *C. ljungdahlii* transformants in

liquid and solid MES medium was routinely carried out in the presence of 5 µg thiamphenicol/mL or 1 mg spectinomycin/mL or both, depending on the plasmid(s) present, unless otherwise indicated.

Genetically engineered *E. coli* TV3007 was used as a control in Western blotting as it had previously been shown capable of synthesizing the heterologous enzymes of the MVA pathway. TV3007 is derived from *E. coli* strain MD12-778 by curing of plasmid pRed-Et. MD12-778 has been described in patent filings US 2013/0273625 and US 2013/0089906.

Preparation of electrocompetent *C. ljungdahlii*

Deoxygenated MES-F medium (15), was inoculated from a frozen stock (MES-F + 25% glycerol) of WT *C. ljungdahlii* in a Type B anaerobic chamber (Coy Laboratory Products, atmosphere 2% H_2, 5% CO_2, 93% N_2) and cultured *i*n an incubator-shaker (110 rpm, Chemglass Life Sciences) at 37°C. Electrocompetent cells were prepared as described in Leang *et al.* (13) except that MES-F liquid medium replaced PETC liquid medium. The cells were suspended in a small volume of ice-cold SMP buffer (13) (typically ~400 µL for 200 mL of initial culture, OD_{600}=0.1) to which was added a 20% volume of 60% DMSO 40% SMP buffer. The suspension was aliquoted in 25 µL volumes into deoxygenated individual cryotubes and quick frozen using liquid N_2. The frozen cultures were stored in a liquid N_2 Dewar and over 4 months' time showed no loss in electrocompetency.

Plasmid amplification

Chemically competent *E. coli* Top10 cells (Invitrogen) were transformed with plasmid DNA according to the manufacturer's instructions, using 15 µg chloramphenicol/mL or 100 µg

spectinomycin/mL as appropriate. Transformants were screened by colony PCR (see Supplementary Files S1 and S2) and correct-sized PCR products were subsequently sequenced. Plasmids were isolated from confirmed transformants using QIAprep Spin Miniprep Kit (Qiagen), eluting with water when used for electroporation into *C. ljungdahlii*.

Plasmid components and transformation

The *Clostridium-E. coli* shuttle plasmids used for synthesis of MVA pathway enzymes in *C. ljungdahlii* were derived from four-part modular shuttle plasmids described by Heap *et al.* (14). A set of these vectors, pMTL82151, 83151, 83353, 84151, 84422 and 85151 were obtained from the laboratory of Prof. Nigel Minton of the University of Nottingham. The genetic elements used were the Gram-negative replicon, ColE1 + tra, the antibiotic resistance markers, *catP* (chloramphenicol) and *aad9* (spectinomycin), multiple cloning sites with P*fdx* (from *C. sporogenes*) and P*thl* (from *C. acetobutylicum*) promoters and RBS and four different Gram-positive replicons, pBP1 repA (*C. botulinum*), pCB102 repH (*C. butyricum*), pCD6 repA (*C. difficile*) and pIM13 rep L (*B. subtilis*).

The constructed plasmids (Table 1) bore the genes encoding the MVA pathway enzymes: the upper pathway genes, *mvaE* and *mvaS* (both from *E. faecalis*) (15), encoding MvaE (a fusion of acetyl-CoA acetyltransferase and 3-hydroxy-3- methylglutaryl-CoA (HMG-CoA) reductase) (38) and MvaS (HMG-CoA synthase), respectively; the lower pathway genes, *mvk* (from *Methanosarcina mazei*), *pmk* and *mvd* (both from *S. cerevisiae*) (15), encoding MvK (mevalonate kinase), PmK (phosphomevalonate kinase) and Mvd (mevalonate diphosphate decarboxylase), respectively. Also incorporated into plasmids were *idi* (from *S. cerevisiae*) and

434 *ispS* (a truncated version from *P. alba*) (15), encoding Idi (isopentenyl pyrophosphate
435 isomerase) and IspS (isoprene synthase), respectively.

436 Plasmid electroporation was similar to Leang *et al.* (13) with some modification. Twenty-
437 five µL of electrocompetent *C. ljungdahlii* cells were thawed on ice in the anaerobic chamber.
438 Two µg of plasmid DNA at ~ 1µg/µL were mixed with the cells. The suspension was then placed
439 in a deoxygenated prechilled (on ice) 1 mm gap electroporation cuvette (Bio-Rad) and
440 electroporated (Bio-Rad Gene Pulser Xcell) at 625 V, with a resistance of 600 Ω and a
441 capacitance of 25 µF. Five hundred µL of deoxygenated pre-chilled MES-F medium were added
442 to the cuvette and then transferred to a serum vial containing 10 mL MES-F. The culture was
443 incubated overnight in an incubator-shaker (37°C at 110 rpm). The cell suspension was spun at
444 4185 × g for 15 min and the pellet resuspended in 200 µL of MES-F. One hundred µL were then
445 spread onto a 100-mm diameter Petri plate (1.5% agar) containing enriched MES-F medium
446 (MES-F supplemented with 10 g proteose peptone/L and 10 g beef extract/L) containing the
447 appropriate antibiotic(s) (5 µg thiamphenicol/mL or 1 mg spectinomycin/mL or both). The agar
448 plates were stored inverted in Oxoid jars (Oxoid Limited), containing anaerobic chamber gas and
449 a palladium catalyst and placed in an incubator at 37°C. Colonies observed after 3 days were
450 restreaked on enriched and unenriched solid MES-F medium containing antibiotic.
451
452 **Plasmid recovery from *Clostridium ljungdahlii***
453 The putative transformants were screened by colony PCR using standard conditions with
454 HotStarTaq Master Mix (Qiagen) and the appropriate primers (see Supplementary Files S1 and
455 S2). Colonies showing the expected-size PCR product were grown in 10 mL liquid MES-F plus
456 antibiotic. Once the cells had reached an OD_{600} of ~0.2, the culture was spun at 4185 × g (15-

min). To each pellet was added 250 µl of Buffer P1 with RNAse A solution (Qiagen) with 50 mg/mL lysozyme (Sigma Life Science). Once resuspended, the cells were removed from the anaerobic chamber and incubated at 37° C for 1 hour. After lysis, plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen) following the Kit instructions. Isolated plasmid was used to back transform chemically-competent *E. coli* Top10 cells. These were PCR colony-screened and sequenced as in Plasmid amplification (above) to verify that the plasmid was fully intact.

Gel electrophoresis and Western blotting

In preparation for Western blotting (see Supplementary File S2), frozen PBS-washed cell pellets were thawed and resuspended in ~1.5 mL PBS (OD_{600}≅33) containing 100 µg/mL phenylmethylsulfonyl fluoride (PMSF). The cell suspensions were passaged three times through a French pressure Mini-Cell (SLM-Aminco) at 138 MPa at ~5°C. Fifteen µL of lysate were mixed with 5 µL of NuPAGE LDS sample buffer (Invitrogen) for each lane and samples were loaded onto Nupage 12% Bis-Tris Gels (Invitrogen).The gels were run using MES SDS Running Buffer (Invitrogen) for ~50 min at constant voltage (200 V).

The proteins were transferred from the gel to a nitrocellulose membrane using an iBlot Gel Transfer Device (Invitrogen). The membrane was then blocked, washed, incubated with primary antibody, washed, incubated with secondary antibody and washed using Blocking solution and Wash buffer (Western Breeze, Invitrogen) all per the manufacturer's instructions.

The primary antibody solution was a 1:1000 (for MvaE, MvaS, Idi and IspS) and 1:2000 (for MvK, PmK and Mvd) dilution of rabbit antiserum in Blocking solution (Western Breeze, Invitrogen). The secondary antibody solution was 2 µg/mL Alexa Fluor 488 goat anti-rabbit IgG

(H+L) (Life Technologies) in Blocking solution. The fluorescent bands were detected in a ProteinSimple instrument (Fluor Chem M) according to the manufacturer's instructions.

The rabbit polyclonal antisera directed against the MVA pathway enzymes plus Idi and IspS were a gift of Yuliya Primak. The enzymes were His-tagged (to aid purification) and synthesized from IPTG-inducible expression plasmids in *E. coli*. The antigens were MvaE and MvaS (*E. faecalis*), Mvk (*M. mazei*) Pmk, Mvd and Idi (*S. cervisiae*) and IspS (*P. alba*).

Detection of mevalonolactone by HPLC

Aliquots of the *C. ljungdahlii* cultures were acidified to convert mevalonate to mevalonolactone (16), spun down (14,000 × g) and the supernatants injected into a series 1100/1200 HPLC (Agilent Technologies) (see Supplementary File S2). An Aminex HPC-87H Ion Exclusion Column (300 mm x 7.8 mm) fitted with a Micro-Guard Cation H Cartridge guard column (both from Bio-Rad) was equilibrated with a 0.01 N H_2SO_4 mobile phase at 60°C at a flow rate of 0.6 mL/min. Sample volumes injected were 10 µL. Mevalonolactone, detected by a Refractive Index Detector (RID, Agilent), thermostated at 55°C, eluted between 18 and 21 min. Standard solutions of mevalonolactone in water at 0.25, 1.0, and 4.0 mg/mL were used to calibrate the measurements.

Detection of mevalonic acid by LC/MS

C. ljungdahlii cultures were centrifuged (10 min, 4800 × g) and 0.5 mL of supernatant and a 0.1 mL methanol pellet extract were combined and buffered with 10 µL of 1 M ammonium acetate (pH=7.0). Ten µL samples were applied to a C18 Synergi MAX-RP HPLC column, fitted with a manufacturer-recommended guard cartridge (Phenomenex). The column was eluted with a

gradient (15) of 15 mM acetic acid + 10 mM tributylamine in water (solvent A) and LC/MS-grade methanol (solvent B), flow rate 0.4 mL/min, column temperature 40° C. Mevalonic acid was detected by mass spectrometry using a TSQ Quantum triple quadrupole instrument (Thermo Scientific). Mevalonic acid was quantified based on intensities of the m/z=147 peak and the 147-59 SRM (Selected Reaction Monitoring) transition compared to a calibration curve.

Syngas Atmosphere

A gassing station was used to replace the chamber gas atmosphere (see above) in septa-sealed culture vessels with the syngas atmosphere (35% CO, 36% H_2, 18% CO_2 and 11% Ar). The septa were pierced with a hyperdermic needle (22G) and the contents of the headspace evacuated using a vacuum pump to a gauge pressure of -75 kPag. The headspace was then filled with N_2 to a gauge pressure of +34 kPag. Evacuation and refilling were repeated three times, but using syngas instead of N_2. The final pressure was +34 kPag after needle withdrawal.

Culture head space detection of CO and CO_2 using gas chromatography

Four mL of *C. ljungdahlii* MES-0F cultures were placed in 16.5 mL septa-sealed vials (Supelco) containing syngas, and shaken at 30° C overnight. After 20 h, 100 µL was withdrawn from the headspace using a gas-tight syringe (Hamilton) and injected into the splitless injection port of a Model 7890B Gas Chromatograph (Agilent) at 150°C using ultra high-purity He as the carrier gas. A CP7429 Select Permanent Gas/CO_2 column (Varian) was run isothermally at 45° C to separate the components of the sample (CO, H_2, CO_2 and Ar), which were detected using a thermal conductivity detector maintained at 200° C.

GC/MS detection of isoprene in culture head space using syringe sampling

One mL samples were taken from the headspace of 10 mL cultures in 16.5 mL Supelco or 20 mL crimp-sealed Agilent vials (as indicated) using a gas-tight syringe (Hamilton). They were injected in splitless mode into the injection port (front inlet temperature 110°C) of a 7890A gas chromatograph (GC, Agilent) coupled to a 7975C mass spectrometer (MS, Agilent, operating in electron ionization mode). The GC column (HP-5ms, Agilent Technologies) was run at 37°C with He (2 mL/min) as the carrier gas. Extracted ion mass spectra (m/z=67) and total ion mass spectra were compared to a 0.3 mL injection of isoprene standard (1090 ppm isoprene in N_2).

GC/MS detection of isoprene in head space using solid-phase microextraction (SPME)

A 75 µm Carboxen/PDMS Fused Silica SPME fiber was used to trap isoprene in the culture vial headspace. The SPME fiber was conditioned by heating at 275^{o}C under helium flow for 15 minutes. The conditioned fiber was then introduced through the septum into a 16.5 mL Supelco vial containing 4 mL of *C. ljungdahlii* culture. The fiber was positioned ~1 cm above the broth, and equilibrated at room temperature for 15 minutes. The fiber was then removed from the vial and immediately inserted into the inlet (at 250^{o}C) of a 7890A/5975C GC/MS instrument (Agilent). The fiber was held in the inlet for 2 minutes. Desorbed material from the fiber was collected onto the chromatographic column (RTX-1, 60 m x 0.320 mm x 3 µm) precooled to -35^{o}C. After desorption, the fiber was removed from the inlet and the column oven was heated at 20^{o}C per minute to a final temperature of 250^{o}C. The inlet was operated in both split and splitless modes. Calibration was with an isoprene standard.

ACKNOWLEDGEMENTS

549 The authors are most grateful to Gail Donaldson, Robert Manning and Judi Lacey for
550 help with designing the plasmid constructs; to James A. Zahn for expert advice on the culturing
551 of acetogens and for building the gassing station; to Dmitri Vaviline and Chuck Powley for some
552 of the GC/MS work and help with HPLC analysis of mevalonate, respectively; to Joseph J.
553 Kozole and James Ngai for help with GC/MS analysis of isoprene; to Yuliya Primak for the gift
554 of the antibodies used for Western blotting; to Yifan Xu, Patricia A. Mauvais and Ann O'Brien
555 for metabolomics analysis; to Gopal Chotani for helpful discussions on the stoichiometry of
556 syngas conversion to isoprene; to Madhukar Dasika, Michael Reichman and Maggie Cervin for
557 helpful discussions on intermediary metabolism and to Tanja Gruber for her support in the
558 publication of this paper.
559

560 **Authors' contributions**

561 BD wrote the manuscript. BD and JF conceived of the experiments and carried out cell
562 culturing, Western blotting, plasmid construction and electroporation, GC-mass spec, GC and
563 HPLC analysis. MS, DW and GW provided the MVA pathway genes, some early plasmids and
564 intellectual input.
565

566 **Competing interests**

567 The authors were all employees of E. I. du Pont de Nemours & Co. at the time that the
568 work described in this manuscript was carried out. The costs of publication are being borne by
569 the DuPont Company.

570 The authors have no non-financial competing interests with regard to this manuscript.

571 All authors read and approved the manuscript.

Funding

This work was funded by the Biochemical Sciences and Engineering division of Central Research and Development and by the Industrial Biosciences division, both of E. I. du Pont de Nemours & Co.

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691

692 **FIGURE LEGENDS**
693

694 Figure 1 - Interaction between the Wood-Ljungdahl pathway, the Embden-Meyerhof-Parnas
695 glycolytic pathway and the Mevalonic Acid Pathway for the synthesis of isoprene.
696 Abbreviations:
697 Wood-Ljungdahl Pathway: FDH (Formate dehydrogenase, which also forms a complex with the
698 Electron bifurcating hydrogenase (EBH), HytA-E) (21), Nfn (NADH-dependent
699 Ferredoxin(red):NADP oxidoreductase) (27), THF (tetrahydrofolate), FTHFS
700 (Formyltetrahydrofolate synthetase), MTHFC (methenyltetrahydrofolate cyclohydrolase),
701 MTHFD (NADP-dependent methylenetetrahydrofolate dehydrogenase), MTHFR
702 (methylenetetrahydrofolate reductase, putative electron-bifurcating complex) (20, 39)(but see
703 (25), MET (methyltetrahydrofolate:corrinoid/iron-sulfur protein methyltransferase, CODH/ACS
704 (Bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase), PTA
705 (Phosphotransacetylase), ACK (Acetate kinase)

706 Embden-Meyerhof-Parnas (EMP) Glycolytic Pathway: FRUpts (Fructose phosphoenolpyruvate-
707 dependent phosphotransferase), HK (Hexokinase), PGI (Phosphoglucose isomerase), PFK
708 (Phosphofructokinase, AYP and PPi-dependent (36)), FBA (Fructose bisphosphate aldolase),
709 DHAP (Dihydroxyacetone phosphate), TPI (Triosephosphate isomerase), GAPDH
710 (Glyceraldehyde phosphate dehydrogenase, NAD and NADP-dependent(34)), PGK
711 (Phosphoglycerate kinase), PGM (Phosphoglycerate mutase), ENO (Enolase), PEP
712 (Phosphoenolpyruvate), PYK (Pyruvate kinase), PFOR (Pyruvate Ferredoxin Oxidoreductase)
713 Mevalonic Acid Pathway: MvaE (acetyl-CoA acetyltransferase/HMG-CoA reductase, NADPH
714 and NADH dependent (28)), MvaS (Hydroxymethylglutaryl-CoA synthase), Mvk (mevalonate
715 kinase), Pmk (Phosphomevalonate kinase), Mvd (Mevalonate diphosphate decarboxylase), Idi
716 (isopentenyl pyrophosphate isomerase), IspS (Isoprene synthase)
717 Rnf Complex: 6-subunit membrane associated ion-motive complex (19)
718 Reactions involving high-energy phosphate bonds are indicated with red arrows.
719

720 Figure 2 - HPLC traces for the detection of mevalanolactone formed by internal esterification of
721 mevalonate, following acidification of fermentation broth. Samples are *C. ljungdahlii* WT
722 (black), pMTL83151 transformant (blue), pJF100 transformant (red) and 0.25 mg/mL
723 mevalonolactone (brown). Ten µL injection of supernatant of acidified fermentation broth. Flow
724 rate 0.6 mL/min. Detection by refractive index detector.
725

726 Figure 3 – GC/MS detection of isoprene in the culture head space for *C. ljungdahlii* cultivated
727 on fructose. *C. ljungdahlii* WT and pJF100 Fdii transformants were cultivated for 1 day at 37°C
728 on MES-F (Table 2 and Supplementary File S2). (A) Gas chromatograms with MS extracted ion

729 detection at m/z=67 for *C. ljungdahlii* WT (black) and pJF100 Fdii transformant plasmid 1
730 (blue), plasmid 3 (red), following injection of a 1 mL sample of culture head space. (B) Gas
731 chromatogram with MS extracted ion detection at m/z=67 for isoprene standard (1090 ppm in
732 N_2) following 300 µL injection. (C) Total ion MS of isoprene standard (1.70-1.80 min) (D) Total
733 ion MS of head space from culture of *C. ljungdahlii* pJF100 Fdii plasmid 1 culture (1.70-1.80
734 min) minus WT spectrum. The OD_{600} of the cultures at the time of headspace measurement was
735 1.788, 0.39 and 2.096 for the WT and pJF100 Fdii transformants with plasmid 1 and plasmid 3,
736 respectively.

737

738 Figure 4 – GC/MS detection of isoprene using SPME microextraction of culture head space for
739 *C. ljungdahlii* cultivated on syngas. *C. ljungdahlii* WT and pJF100 Fdii N-term His tagged IspS
740 transformant were cultivated for 20 h at 30°C on MES-0F plus syngas (Table 2 and
741 Supplementary File S2). After starting at 0.5, the final OD_{600} was 0.888 and 1.034, respectively.
742 The headspace was first sampled for GC determination of CO and CO_2 (see Table 3) and then
743 separately sampled using SPME for isoprene content (see Table 2). (A) Gas chromatogram of the
744 SPME desorbed material with MS total ion detection for the WT (black) and the transformant
745 (blue). The isoprene retention time is 10.25 min as determined by an isoprene standard (not
746 shown). (B) Gas chromatogram with MS detection of isoprene using extracted ion mode (m/z =
747 67, average of 66.70 to 67.70) and splitless injection for the WT (black) and transformant (blue).
748 (C) MS total ion mass spectrum at 10.251 to 10.26 min retention time for the transformant
749 compared to the total ion mass spectrum (D) of an isoprene standard.

750

751 Figure 5 – Cultivation on syngas (30°C) and production of mevalonate in syngas-adapted *C.*
752 *ljungdahlii* WT and pJF100#2-transformant. The transformant was initially cultivated in MES-
753 0.1 F + 5µg thiamphenicol/mL under syngas and diluted 10-fold to 0.1 OD_{600} in MES-0F + 5 µg
754 thiamphenicol/mL under syngas. After growing to an OD_{600} of 2.368, the cells were pelleted and
755 resuspended in fresh MES-0F medium + 5 µg thiamphenicol/mL under syngas. Samples were
756 taken on Day 7 of the first passage (■) on MES-0F under syngas and on Days 1, 2 and 3 of the
757 second passage (□) on MES-0F under syngas. The mevalonate (mva) concentrations were
758 determined by LC/MS. The mevalonate concentration of the syngas-adapted WT *C. ljungdahlii*
759 ((▲), 7-day MES-0F culture under syngas) is equal to that of the MES-0F medium alone. The
760 cell density was followed by OD_{600}.

761

762 **TABLES**

763 Table 1 – Plasmids used and constructed in this work

Plasmid	**Gram+ replicon**	**Marker**	**Gram- replicon**	**Promoter + MVA pathway genes**
pMTL83151	pCB102	*catP*	ColE1	
pMCS278	pIM13	*ermB*	ColE1	P*fdx* + *mvaE* + *mvaS*
pJF100	pCB102	*catP*	ColE1	P*fdx* + *mvaE* + *mvaS*
pMCS337 (pDW253)	pIM13	*catP*	ColE1	Awo1181 + *ispS* + *idi*
pJF100 Fdii	pCB102	*catP*	ColE1	P*fdx* + *ispS* + *idi*
pJF100 Fdii producing N-term His-tagged IspS	pCB102	*catP*	ColE1	P*fdx* + N-His-*ispS* + *idi*
pJF100 Fdii producing C-term His-tagged IspS	pCB102	*catP*	ColE1	P*fdx* + C-His-*ispS* + *idi*
pJF101	pBP1	*catP*	ColE1	P*thl* + *ispS* + *idi*
pJF102	pCB102	*aad9*	ColE1	P*fdx* + *mvk* + *pmk* + *mvd*
pJF200	pCB102	*catP*	ColE1	Awo1181 + *ispS* + *idi*

764

765

766 Table 2 – Isoprene determinations for *C. ljungdahlii* strains

Isoprene Determination	Strain	OD_{600}	Isoprene in headspace (ng/mL)	Mass isoprene per volume broth (ng/mL)	Isoprene ratio Transformant to Wild Type
Fructose to isoprene on MES-F, 1 d, 37°C	WT	1.788	~1.2	~1.2	4-5
	pFdii transformant plasmid 3	2.096	5	5	
Syngas to isoprene on MES-0F, 20 h 30°C 1st SPME extraction,	WT	0.500 → 1.034	0.077	0.24	6.2
	pFdii N-term His-tagged IspS transformant	0.500 → 0.888	0.48	1.50	
	Medium additive				Isoprene ratio mva to no mva added
Mevalonate (mva) to isoprene on MES-F, 15.5 h, 37°C	pJF101 + pJF102 #4 transformant No added mva	0.4 → 1.25	~14	~9	~10
	pJF101 + pJF102 #4 transformant 10, 20 and 40 mM mva	0.4 no growth	140	91	

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768

769 Table 3 – Consumption of CO and mole ratio of CO_2 produced/CO consumed in syngas-

770 cultivated WT and *C. ljungdahlii* transformants

Syngas-adapted WT and transformed strains	% of CO consumed	OD_{600} change	Mole ratio $\frac{CO_2 \text{ produced}}{CO \text{ consumed}}$ (average of two measurements)
pJF100 - 26 h in MES-0F	52.65 ± 1.64	0.500 to 0.838	0.237 ± 0.090 (3 measurements)
pJF100 - 26 h in MES-0F	51.90 ± 0.14	0.500 to 0.806	0.185 ± 0.010 (2 measurements)
pJF100 Fdii N-term His-tagged Isps - 20 h in MES-0F	42	0.500 to 0.888	0.44 ± 0.07
pJF100 Fdii C-term His-tagged Isps -20 h in MES-0F	52	0.500 to 0.952	0.34 ± 0.35
WT in MES-0F -20 h	58	0.500 to 1.034	0.41 ± 0.12

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