1 **Pantothenate auxotrophy in *Zymomonas mobilis* ZM4 is due to**

2 **a lack of aspartate decarboxylase activity**

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Abstract

The bacterium *Zymomonas mobilis* naturally produces ethanol at near theoretical maximum yields, making it of interest for industrial ethanol production. *Z. mobilis* requires the vitamin pantothenate for growth. Here we characterized the genetic basis for the *Z. mobilis* pantothenate auxotrophy. We found that this auxotrophy is due to the absence of a single gene, *panD*, encoding aspartate-decarboxylase. Heterologous expression of *Escherichia coli* PanD in *Z. mobilis* or supplementation of the growth medium with the product of PanD activity, β-alanine, eliminated the need for exogenous pantothenate. We also determined that *Z. mobilis* IlvC, an enzyme better known for branched-chain amino acid synthesis, is required for pantothenate synthesis in *Z. mobilis*, as it compensates for the absence of PanE, another pantothenate synthesis pathway enzyme. In addition to contributing to an understanding of the nutritional requirements of *Z. mobilis*, our results have led to the design of a more cost-effective growth medium.

24 **Introduction**

25 *Zymomonas mobilis* is a bacterium best known as a potential rival to the ethanol-
26 producing yeast *Saccharomyces cerevisiae*. *Z. mobilis* ferments glucose into ethanol at 97% of
27 the theoretical maximum yield, produces ethanol 3 – 5 times faster than yeast on a per cell basis,
28 and produces less residual biomass than yeast (Jeffries 2005). Unlike yeast, *Z. mobilis* can also
29 use inexpensive N_2 gas a nitrogen source, raising the possibility to grow *Z. mobilis* on nitrogen-
30 poor cellulosic feedstocks without the need for expensive undefined nitrogen supplements, such
31 as corn steep liquor (Kremer *et al.* 2015). However, these undefined supplements can also satisfy
32 vitamin requirements (Lawford and Rousseau 1997). The vitamin pantothenate (vitamin B5), a
33 precursor to coenzyme-A, is required by all *Zymomonas* isolates characterized to date (Belaich
34 and Senez 1965; De Ley and Swings 1976; Nipkow, Beyeler and Fiechter 1984; Galani, Drainas
35 and Typas 1985; Lawford and Stevnsborg 1986; Cross and Clausen 1993). Elimination of this
36 auxotrophy, combined with utilizing N_2 as a nitrogen source, would circumvent the need for
37 nutrient rich supplements altogether for *Z. mobilis* growth on cellulosic feedstocks.
38

39 Herein, we describe the genetic basis for panthothenate auxotrophy in the most
40 commonly used *Z. mobilis* research strain, ZM4 (Seo *et al.* 2005; Skerker *et al.* 2013). A
41 comparative genomics analysis of the seven sequenced *Z. mobilis* isolates indicated that the
42 entire pantothenate synthesis pathway (depicted in Fig. 1) is missing in two isolates while the
43 other five strains, including ZM4, are only missing *panD* and *panE* (Fig. 2). We found that β-
44 alanine could support ZM4 growth in place of pantothenate. In support of this observation,
45 expression of a heterologous PanD, the enzyme producing β-alanine from aspartate (Fig. 1),
46 eliminated the pantothenate auxotrophy. We also discovered that the lack of *panE* was

47 inconsequential for pantothenate synthesis as the activity was compensated for by native IlvC, an
48 enzyme better known for its role in branched-chain amino acid synthesis. Our results indicate
49 that β-alanine can serve as a less expensive growth supplement in place of pantothenate and that
50 heterologous expression of a single gene, PanD, is sufficient to eliminate the pantothenate
51 auxotrophy.
52
53 **Materials and Methods**
54 **Strains and growth conditions.** All strains are described in Table 1. *Z. mobilis* ZM4 (ATCC
55 31821) and the IlvC transposon mutant (ZMO1141::Tn5; UP33_A10), a mutant in which the
56 native *ilvC* is interrupted by a transposon, were kindly given to us by J. M. Skerker and A. P.
57 Arkin, UC Berkeley (Skerker *et al.* 2013). Transposon mutagenesis and identification of the Tn5
58 insertion site in the ZMO1141::Tn5 mutant is described elsewhere (Skerker *et al.* 2013). For
59 cloning experiments, *Z. mobilis* was grown in aerobic YPG (1% yeast extract, 2% peptone, 2%
60 glucose) or plated on YPG agar (1.5% agar). All growth experiments were conducted in 10 ml of
61 a chemically-defined growth medium (ZYMM) in anaerobic test tubes with shaking at 150 rpm
62 to avoid cell settling as described (Kremer *et al.* 2015). Where indicated, calcium pantothenate
63 and β-alanine were added at a final concentration of 100 nM each, and branched chain amino
64 acids (isoleucine, leucine, and valine) were added at a final concentration of 0.5 mM each. Media
65 were made anaerobic by bubbling with Ar gas and then sealing tubes with rubber stoppers (Geo-
66 Microbial Technologies, Ochelata, OK) and aluminum crimps. No CO_2 produced by *Z. mobilis*
67 was released during the course of an experiment. Starter cultures were inoculated with a single
68 colony from YPG agar, and then a 1% inoculum was transferred to test cultures. *Escherichia coli*
69 strains used for cloning were grown in LB broth or on LB agar. Where noted, tetracycline was

70 used at 5 μg/ml for *Z. mobilis* and at 15 μg/ml for *E. coli*, and kanamycin was used at 100 μg/ml

71 for *Z. mobilis*. *Z. mobilis* was grown at 30°C and *E. coli* was grown at 37°C.

72 **Construction of *Z. mobilis* gene expression vectors.** All plasmids and primers are described in

73 Table 1. All enzymes and competent cells were used according to the manufacturer's

74 instructions. To express *E. coli* PanD in *Z. mobilis*, the ZM4 *panC* promoter (P*panC*) was first

75 amplified from ZM4 genomic DNA using primers to introduce NdeI and SacI restriction sites

76 upstream and downstream of the promoter, respectively. The PCR product was digested with

77 NdeI and SacI (NEB, Ipswich, MA) and then ligated into pSRKTc (Khan *et al.* 2008) that had

78 been digested with the same enzymes. The ligation reaction was used to transform chemically

79 competent *E. coli* NEB10β (NEB) and then cells were plated on selective media. Transformants

80 were screened for pSRKTc with the P*panC* insert using colony PCR and the correct sequence

81 was confirmed by Sanger sequencing. Next, the *E. coli panD* gene was amplified from *E. coli*

82 MG1655 genomic DNA using primers to introduce SacI and XhoI restriction sites upstream and

83 downstream of the gene, respectively. The gene was then inserted into the pSRKTc_PpanC

84 plasmid downstream of the P*panC* promoter using the same procedure as above.

85 All plasmids were transformed into ZM4 by electroporation. ZM4 was first made electro-

86 competent by growing cells to mid-exponential phase in 100 ml YPG, harvesting by

87 centrifugation, washing three times in 10 ml of 10% ice-cold glycerol, and resuspending in 1 ml

88 10% ice-cold glycerol. Fifty μl aliquots were frozen in an ethanol-dry ice bath and then stored at

89 -80°C. Electroporation was carried out with a BioRad MicroPulse electroporator (Hercules, CA),

90 using the manufacturer's pre-programed 'Ec1' setting (single 1.8 kV pulse for > 5.5 ms), in 1mm

91 electroporation cuvettes. Electroporated cells were allowed to recover in 5 ml YPG for at least 18

92 hours at 30°C without shaking before plating onto selective media.

93 **Analytical techniques.** Cell densities were monitored by optical density at 660nm (OD_{660}) as
94 described (Gordon and McKinlay 2014). Glucose and ethanol were quantified using a Shimadzu
95 (Kyoto, Japan) high performance liquid chromatograph as described (McKinlay, Zeikus, and
96 Vieille 2005).
97
98 **Results**
99 **Comparison of pantothenate synthesis operons in *Z. mobilis* isolates.** Pantothenate
100 auxotrophy is a common attribute among *Z. mobilis* isolates (Belaich and Senez 1965; De Ley
101 and Swings 1976; Nipkow, Beyeler and Fiechter 1984; Galani, Drainas and Typas 1985;
102 Lawford and Stevnsborg 1986; Cross and Clausen 1993). In fact, a survey of 38 *Zymomonas* sp.
103 isolates found that all strains required pantothenate (De Ley and Swings 1976). In ZM1, a strain
104 for which no genome sequence is publically available, heterologous expression of both *panD* and
105 *panE* eliminated the auxotrophy (Tao, Tomb and Viitanen 2014). To gauge if a lack of *panD* and
106 *panE* might similarly explain the pantothenate auxotrophy in other *Z. mobilis* isolates, we used
107 BLAST (Altschul *et al.* 1990) to look for protein sequences similar to *E. coli* MG1655 PanB,
108 PanC, PanD, and PanE in the seven sequenced *Z. mobilis* strains (Seo *et al.* 2005; Kouvelis *et al.*
109 2009, 2011; Pappas *et al.* 2011; Desiniotis *et al.* 2012). None of the seven strains had homologs
110 of *panD* or *panE*. Five of the seven strains have putative *panB* and *panC* genes in a region of
111 conserved gene arrangement, or synteny (Fig. 2). One of these five strains, ZM4, had an
112 additional *panBC* gene pair (ZMO1952 and ZMO1971, respectively) 22.4 kb away from the
113 *panBC* pair in this region (ZMO1970 and ZMO1954, respectively), but otherwise there was no
114 syntenty between the two regions. The other two strains, ATCC 29191 and ATCC 29192, lacked

115 genes with any significant sequence similarity to *panB* and *panC* but otherwise showed synteny
116 in this region (Fig. 2).
117
118 ***Z. mobilis* ZM4 is auxotrophic for β-alanine but not for pantoate.** The absence of *panD* and
119 *panE* suggested that *Z. mobilis* strains having *panB* and *panC* should be incapable of making
120 both β-alanine and pantoate (Fig. 1). In examining the pantothenate synthesis pathway in the
121 metabolism database MetaCyc (Caspi *et al.* 2014), we noticed that other bacteria can make
122 pantoate using IlvC, an enzyme with a broad substrate range that is better known for
123 acetohydroxy acid isomeroreductase activity in branched-chain amino acid synthesis. In fact,
124 IlvC and PanE have redundant pantoate synthesis activity in *E. coli* (Elischewski, Pühler and
125 Kalinowski 1999) and IlvC is the only enzyme responsible for pantoate synthesis in
126 *Corynebacterium glutamicum* (Merkamm *et al.* 2003). All seven *Z. mobilis* genomes encode a
127 protein with 37% identity to the *E. coli* IlvC. If *Z. mobilis* can make pantoate using IlvC then the
128 pantothenate auxotrophy could be due to an inability to synthesize β-alanine alone. We therefore
129 tested whether *Z. mobilis* could grow when supplied with β-alanine in place of pantothenate.
130 Growth trends were similar when either β-alanine or pantothenate were provided (Fig. 3A),
131 whereas no growth was observed when both supplements were omitted (Fig. 3A). Providing β-
132 alanine in place of pantothenate also had no effect on the ethanol yield (Fig. 3B).
133
134 Since β-alanine can substitute for pantothenate as an essential growth supplement, we
135 hypothesized that the auxotrophy could be eliminated by expressing PanD, the enzyme that
136 produces β-alanine by decarboxylating aspartate (Fig. 1). To test this hypothesis, we constructed
137 a plasmid for expressing the *E. coli panD* gene under control of the *Z. mobilis panC* promoter.

138 This plasmid allowed ZM4 to grow in a medium lacking both pantothenate and β-alanine,

139 whereas an empty vector did not (Fig. 4). We conclude that the *Z. mobilis* ZM4 pantothenate

140 auxotrophy is due to a lack of β-alanine-producing aspartate decarboxylase (PanD) activity and,

141 by extension, that ZM4 has the native capacity to synthesize pantoate.

142

143 ***Z. mobilis* ZM4 IlvC is responsible for pantoate synthesis.** As noted above, IlvC, an enzyme

144 involved in branched-chain amino acid synthesis, can substitute for the pantoate synthesis

145 activity of PanE in other bacteria (Elischewski, Pühler and Kalinowski 1999; Merkamm *et al.*

146 2003). The ZM4 *ilvC* gene, ZMO1141, is located 872.6 kb away from the *panBC* pair shown in

147 Figure 2. To test whether IlvC is responsible for pantoate synthesis in ZM4, we examined the

148 growth requirements of a mutant with a transposon inserted into the *ilvC* gene, IlvC::Tn, for

149 pantothenate and β-alanine. The IlvC::Tn mutant could only grow when both pantothenate and

150 branched-chain amino acids were provided (Fig. 5). Unlike for WT ZM4, supplying β-alanine in

151 place of pantothenate did not support IlvC::Tn mutant growth. The strict requirement for both

152 pantothenate and branched-chain amino acids by the IlvC::Tn mutant indicates that IlvC is

153 required for *de novo* synthesis of both pantothenate and branched-chain amino acids in *Z.*

154 *mobilis*. (Fig. 5).

155

156 **Discussion.** We have demonstrated that the pantothenate auxotrophy in *Z. mobilis* ZM4 is due to

157 the absence of PanD, encoding aspartate decarboxylase (Fig 4). The absence of *panE* does not

158 factor into the auxotrophy, as its absence is compensated for by the activity of IlvC (Fig 5),

159 similar to what has been observed in some other bacteria (Elischewski, Pühler and Kalinowski

160 1999; Merkamm *et al.* 2003). A patent previously reported that expression of heterologous *panD*

161 and *panE* in ZM1 eliminated the pantothenate auxotrophy; however, the effects of expressing
162 each gene individually was not tested (Tao, Tomb and Viitanen 2014). While the ZM1 genome
163 sequence is not publically available, microarray analysis has shown it to be highly similar to the
164 ZM4 genome sequence (Seo *et al.* 2005). ZM1 is missing 54 genes that are present in ZM4,
165 including the possible second *panB* copy (ZMO1952), but IlvC (ZMO1141) was not among the
166 list of missing genes in ZM1 (Seo *et al.* 2005). Thus, it might only be necessary to express *panD*
167 in ZM1 to eliminate the pantothenate auxotrophy. The same is likely true for other *Z. mobilis*
168 strains that encode *panB* and *panC* (Fig 2). Separately, we found that the product of PanD
169 activity, β-alanine, could substitute for pantothenate in supporting ZM4 growth in a defined
170 medium (Fig 3). β-alanine costs less than a tenth of that of pantothenate and thus can be viewed
171 as a more cost-effective supplement for *Z. mobilis* defined media.
172
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177
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223

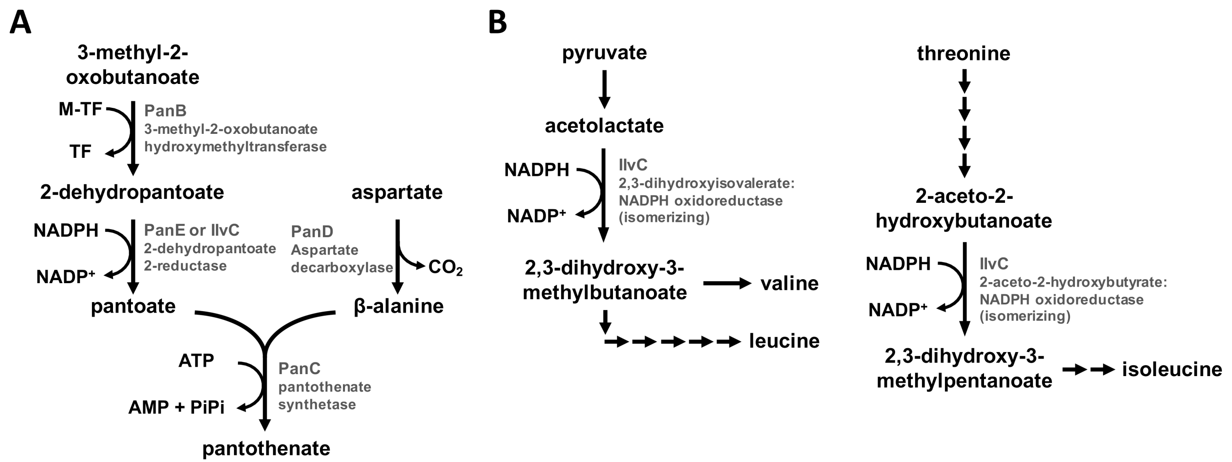
224 **Table 1. Strains, plasmids, and primers**.

Strain, designation in text	Genotype	Source
ATCC 31821, ZM4	Wild-type	(Skerker *et al.* 2013)
UP33_A10, IlvC::Tn5	ZMO1141::Tn5, Kn^R Tn5 transposon inserted after the 324th nucleotide of the 1020 nucleotide gene.	(Skerker *et al.* 2013)
Plasmid		
pSRKTc	Empty vector, Tc^R	(Khan *et al.* 2008)
pSRKTc_PpanC_EcPanD	*E. coli* PanD expression vector, Tc^R	This study
Primer	**Sequence (5' → 3'); Restriction site**	
PpanC_For_NdeI	agcatatggatatttcctttacggcctttg	This study
PpanC_Rev_SacI	atgagctctccatttctgtctctatgaatgact	This study
panD_For_ SacI	aggagctcatgattcgcacgatgctg	This study
panD_Rev_XhoI	agctcgagcggattcgctggagac	This study

225

226

227 ## Figure legends



228

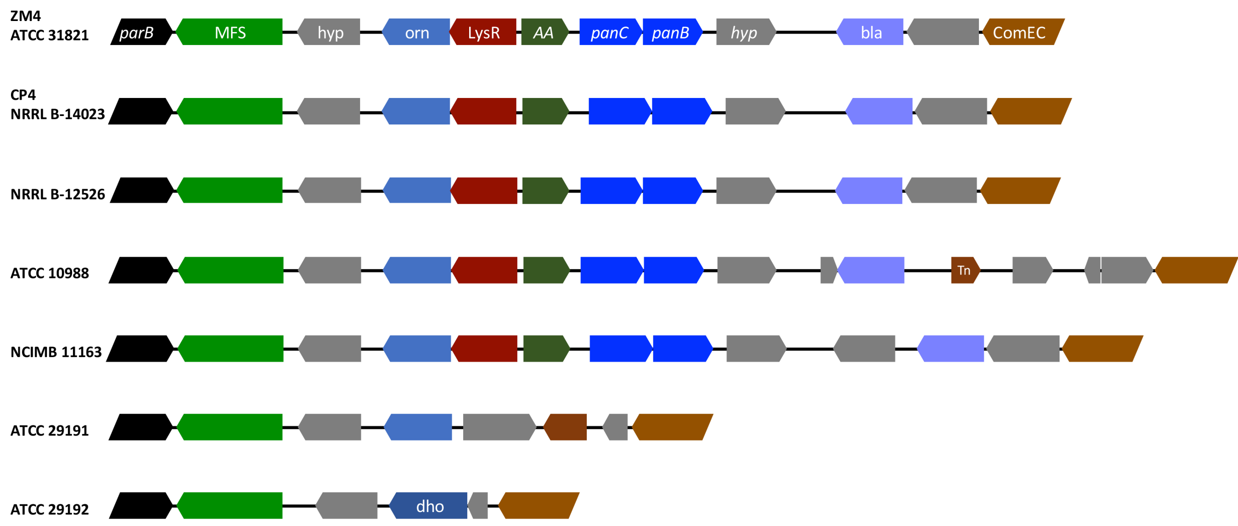
229 **Fig 1. Typical pantothenate (A) and branched-chain amino acid (B) synthesis pathways**

230 **based on those found in *E. coli*.** (**A**) 3-Methyl-2-oxobutanoate is derived from valine. M-TF,

231 5,10-methylene-tetrhydrofolate; TF, tetrahydrofolate. (**B**) Only the reactions that IlvC

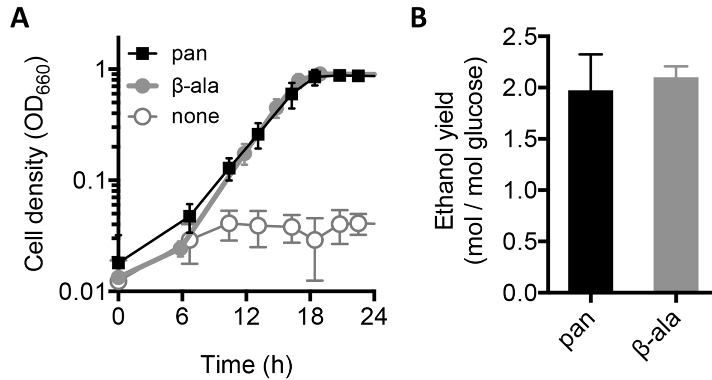
232 participates in are shown in detail.

233



234

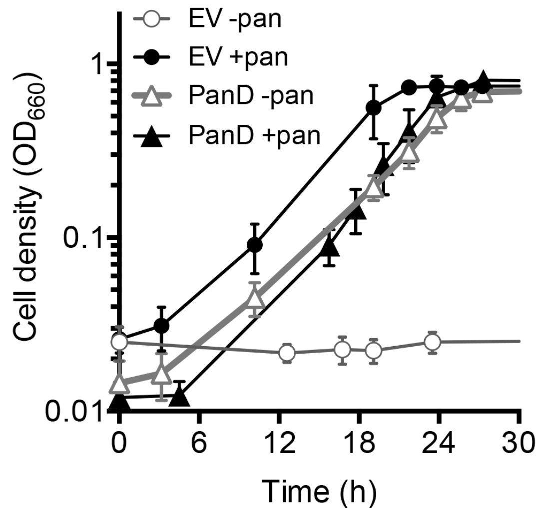
235 **Fig 2. Synteny of *panB* and *panC* genomic regions in the seven sequence *Z. mobilis* strains.**
236 Arrow color indicates gene function. Black, *parB*; Light green, major facilitator superfamily
237 transporter; grey, hypothetical protein; Blue, enzymes: orn, ornithine cyclodeaminase; dho,
238 dihydroorotate oxidase; bla, β-lactamase; Red, LysR-family regulator; Dark green, amino acid
239 transporter (AA); light brown, ComEC competence protein; dark brown, transposon (Tn). In ZM4,
240 the locus tags for *panB* and *panC* are ZMO1970 and ZMO1954, respectively. The *panBC*
241 homologs, respectively ZMO1952 and ZMO1971, are located 22.4 kb away.



242

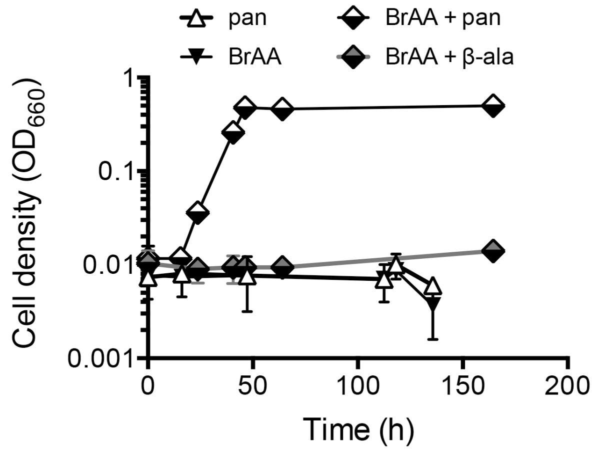
243 **Fig 3. β-alanine (β-ala) can substitute for pantothenate (pan) to support ZM4 growth.** (**A**)
244 Lines represent ZM4 growth curves in a chemically-defined medium with the specified
245 supplement. (**B**) Ethanol yields from culture conditions used in panel A. **(A, B)** Error bars = SD;
246 n=3; error bars are smaller than symbols in some cases.

247



248

249 **Fig. 4. PanD activity allows ZM4 to grow without pantothenate.** Lines represent growth curves
250 for ZM4 with and without *E. coli* PanD in a chemically-defined medium with the specified
251 supplement. EV, empty vector (pSRKTc); PanD, PanD expression vector
252 (pSRKTc_PpanC_EcPanD); pan, pantothenate. All cultures contained tetracycline. Error bars =
253 SD; n=3; error bars are smaller than symbols in some cases.



254

255 **Fig 5. IlvC is required for synthesis of both branched-chain amino acids and pantothenate**
256 **in ZM4.** Lines represent growth curves for the IlvC::Tn mutant in a chemically-defined medium
257 with the specified supplement. BrAA, branched-chain amino acids (isoleucine, leucine, and
258 valine); pan, pantothenate; β-ala, β-alanine. Error bars = SD; n=3; error bars are smaller than
259 symbols in some cases.