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Lactobacillus reuteri DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation
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1 **Title:** ***Lactobacillus reuteri DSM 20016*** **produces cobalamin-dependent diol dehydratase in**
2 **metabolosomes and metabolises 1,2-propanediol by disproportionation**

3

4

5 **running title**

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7 ***L. reuteri*** **metabolosomes and 1,2-propanediol metabolism**

8

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23 **Abstract**

24 A *Lactobacillus reuteri* strain isolated from sourdough is known to produce the vitamin
25 cobalamin. The organism requires this for glycerol co-fermentation by a cobalamin-dependent
26 enzyme usually termed glycerol dehydratase in the synthesis of the antimicrobial substance
27 reuterin. We show that the cobalamin-synthesizing capacity of another *L. reuteri* strain (20016,
28 the type strain, isolated from the human gut and recently sequenced as F275) is genetically and
29 phenotypically linked, as in *Enterobacteriaceae*, to the production of a cobalamin-dependent
30 enzyme which is associated with a bacterial microcompartment (metabolosome) and known as
31 diol dehydratase. We show that this enzyme allows *L. reuteri* to carry out a disproportionation
32 reaction converting 1, 2-propanediol to propionate and propanol. The wide distribution of this
33 operon suggests it is adapted to horizontal transmission between bacteria. However, significant
34 genetic and phenotypic differences are noted in a *Lactobacillus* background compared to
35 *Enterobacteriaceae*. Electron microscopy reveals that the bacterial microcompartment in *L.*
36 *reuteri* occupies a smaller percentage of the cytoplasm than in Gram-negative bacteria. DNA
37 sequence data shows evidence of a different regulatory control mechanism from that in Gram-
38 negative bacteria with the presence of a catabolite responsive element (cre sequence)
39 immediately upstream of the *pdu* operon encoding diol dehydratase and metabolosome structural
40 genes in *L. reuteri*. The metabolosome-associated diol dehydratase we describe is the only
41 candidate glycerol dehydratase present on inspection of the *L. reuteri* F275 genome sequence.
42

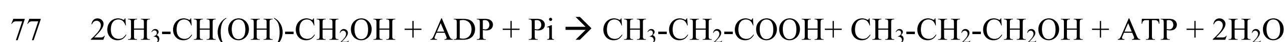
43 **Introduction**

44 *Lactobacillus reuteri* is a probiotic bacterium able to colonise the gastrointestinal tract of a wide
45 variety of mammals and birds (12). It produces an antimicrobial agent (45) (reuterin) by

46 fermentation of glycerol. *Lactobacillus sp* (including *L. reuteri*) cannot grow on glycerol as a sole
47 carbon source, but *L. reuteri* can use beta-hydroxypropionaldehyde (3-HPA) it derives from
48 glycerol as a hydrogen acceptor in fermentation of other carbohydrates including glucose and
49 lactose (44). Unlike most lactobacilli, *L. reuteri* grown in this way on glycerol and another
50 carbohydrate excrete large amounts of reuterin, consisting of an equilibrium mixture of different
51 monomeric and dimeric forms of 3-HPA which has been shown to correspond to reuterin (46,
52 56). There is an optimal ratio of glycerol and glucose for maximal 3-HPA production, and if
53 excess glucose is supplied, 3-HPA is further reduced to 1,3-propanediol by an 1,3-
54 propanediol:NAD oxidoreductase (23) (Figure 1).
55
56 It has been suggested there are two distinct cobalamin-dependent dehydratase enzymes in *L.*
57 *reuteri* that can produce HPA from glycerol (47). Certainly, in *Klebsiella pneumoniae* (49) and a
58 variety of other *Enterobacteriaceae* (13, 51), two different isofunctional cobalamin-dependent
59 enzymes, glycerol dehydratase (EC 4.2.1.30) and diol dehydratase (EC 4.2.1.28) can catalyse the
60 key reaction of glycerol dehydration to 3-HPA (Figure 1) (13, 52). They can also both convert a
61 different substrate, 1,2-propanediol (1,2-PD), to propionaldehyde. Diol dehydratase genes are
62 associated in many *Enterobacteriaceae* with a functional cobalamin synthesis pathway (21) and
63 the production of a proteinaceous cellular microcompartment localising the active enzyme,
64 resembling the carboxysome containing ribulose 1,5-bisphosphate carboxylase/oxygenase
65 (RuBisCO) in autotrophic bacteria (10). This structure in heterotrophic *Enterobacteriaceae* has
66 been termed an enterosome (11), or even carboxysome (29) (based on a hypothesis that carbon
67 dioxide fixation may also occur in these heterotrophic bacteria). Generic terms such as bacterial
68 microcompartment (16), or metabolosome (10) have also been proposed for all such structures,

and we use the term metabolosome. Phylogenetic analysis suggests that, despite their size and complexity, linked cobalamin synthesis and metabolosome synthesis operons are frequently horizontally transmitted (21).

In *Enterobacteriaceae* like *Salmonella* (27) and *Klebsiella* (50), the metabolosome-associated propanediol utilisation operon specifies enzymes for a dismutation that converts 1,2-propanediol (via propionaldehyde) to approximately equal amounts of n-propanol (reduced) and propionate (oxidised). ATP is produced via substrate level phosphorylation.



$$2CH_3\text{-}CH(OH)\text{-}CH_2OH + ADP + Pi \rightarrow CH_3\text{-}CH_2\text{-}COOH + CH_3\text{-}CH_2\text{-}CH_2OH + ATP + 2H_2O$$

Because, unlike most other lactic acid bacteria, *L. reuteri* CRL1098 (48) (a lactic acid bacterium isolated from sourdough) produces cobalamin due to the presence of a multigene operon resembling that present in *Salmonella* and *Listeria* (37), we hypothesized that, as in *Enterobacteriaceae* and other Gram negative bacteria, this capacity was due to horizontally acquired genes which also specified production of a metabolosome containing a diol dehydratase enzyme. No demonstration of 1,2-PD utilisation or bacterial microcompartment production has previously been reported in *L. reuteri* strains. We show that in *L. reuteri* 20016 (the type strain, originally isolated from human faeces) a bacterial microcompartment is present and inducible 1,2-PD utilisation is present with disproportionation to propionate and propanol. Cobalamin is also synthesized. Preliminary analysis of genome sequence data shows the presence of linked cobalamin synthesis and propanediol utilisation operons as in Gram-negative bacteria, with a distinct Gram positive cre element potentially regulating gene transcription in a *Lactobacillus* background.

92

93 **Materials and methods**

94 **Bacteria and growth conditions**

95 *Lactobacillus reuteri* NCDO 2589 was obtained from the National Collection Dairy Organisms,
96 Reading, UK now NCIMB Ltd, Aberdeen, UK). This is also known as DSM 20016, the type
97 strain, and F275 which has recently been sequenced, and was originally isolated from human
98 faeces. *Lactobacillus reuteri* 100-23 was obtained from Professor Gerald Tannock (University of
99 Otago, New Zealand). This strain is also known as DSM 17509 and has been sequenced. It was
100 originally isolated from the digestive tract of a rat (58). *L. reuteri* 100-23 was employed only as a
101 negative control in the propanediol metabolism assay and growth curves. All other references to
102 *L. reuteri* in the manuscript refer to *L. reuteri* DSM 20016. *L. reuteri* strains were grown in MRS
103 (de Man-Rogosa-Sharpe) broth overnight (de Man et al., 1960) containing 15 mM glucose at
104 37°C without shaking. To test for reuterin production by acrolein-based quantitation of HPA,
105 MRS broth containing 250 mmol-1 glycerol as well as glucose was used with anaerobic
106 incubation for 24 hours at 37°C. For the isolation of metabolosomes and for the purification of
107 the enzyme diol dehydratase *L. reuteri* was grown in conical flasks containing MRS broth
108 supplemented with 50 mM 1, 2-PD and 15 mM glucose at 37°C for 36 h. For the detection of
109 metabolosomes using transmission electron microscopy *L. reuteri* was grown in MRS broth
110 containing 65 mM 1, 2-PD with and without 15 mM glucose at 37°C for 18 h. For dismutation of
111 1,2-PD, *L. reuteri* was grown in modified MRS (MRS-MOD) medium, pH5.7 (19) supplemented
112 with 40 mM 1,2-PD without glucose, at 37°C in anaerobic conditions for 8 days. MRS-MOD is a
113 complex medium containing per litre: 5g bacto-peptone, 4g Lab-Lemco (Oxoid), 2 g yeast

114 extract, 0.5 ml Tween 80, 1.0 g K_2HPO_4, 3.0 g $NaH_2PO_4H_2O$, 0.6 g CH_3COONa, 0.3 g

115 $MgSO_4$.7H2O, 0.04g $MnSO_4H_2O$.

116 **Isolation of metabolosomes and protein separation**

117 Protein preparations were initially carried out by a modification of a published procedure (14).
118 Briefly, *L. reuteri* grown in MRS broth containing 1, 2-PD and glucose was harvested by
119 centrifugation at 4000 x g for 10 min. The pelleted cells were washed with 300 ml lysozyme
120 buffer (50 mM Tris-Cl, 0.6 M sucrose, 5 mM EDTA, 0.2% 1, 2-propanediol [pH 8.0]) and
121 resuspended in 30 ml of the same buffer containing 5 mg/ml lysozyme, incubated at 37°C for 2 h
122 with occasional agitation. All further steps were carried out at 4^oC. Lysozyme-treated cells were
123 pelleted by centrifugation at 7,500 x g for 15 min, washed with lysozyme buffer and resuspended
124 in sonication buffer (50 mM Tris-Cl, 2 mM EDTA, 0.2% 1, 2-propanediol, pH 8.0) at
125 approximately 0.1 g wet cell mass per ml. Cells were lysed by sonication, 4 x 120 s bursts with 1
126 min cooling intervals on ice, using SoniPrep 150 (MSE UK Ltd). The crude cell extract obtained
127 by sonication was mixed with an equal volume of BPER-II (Pierce, USA) supplemented with 400
128 mM NaCl and 20 mM $MgCl_2$ and incubated for 30 min at 4°C with shaking. Unlysed cells were
129 removed by centrifugation at 12,000 x g for 10 min. The resulting supernatant was subjected to
130 ultracentrifugation (Beckman SW-40Ti rotor) at 49,000 x g for 90 min. The crude protein pellet
131 was resuspended in 5 ml of Tris-EDTA-$MgCl_2$-propanediol (TEMP) buffer (50 mM Tris-Cl, 1
132 mM EDTA, 10 mM $MgCl_2$, 0.2% 1, 2-propanediol, pH 8.0) and clarified by centrifugation at
133 12,000 x g for 10 min. The clarified preparation was layered on to 4x 11 ml, 35% to 65% w/v
134 sucrose density gradients and centrifuged at 30,000 x g for 16 h. Fractions including the pellet
135 were taken and assayed for diol dehydratase activity. Dehydratase positive fractions were
136 retained in sucrose buffer, and the pellet was resuspended in 1 ml of TEMP buffer and clarified

137 by centrifugation before electron microscopy. Protein preparations for peptide fingerprinting
138 were carried out by cell sonication as above (omitting lysozyme and admixture with B-PER) with
139 subsequent fractionation of the total crude cell lysate by sucrose density gradient centrifugation,
140 selecting diol dehydratase positive fractions for SDS-PAGE separation.

141 **Protein separation**

142 50 μg aliquots of extracted protein was separated by SDS-PAGE using a 12.5% polyacrylamide
143 gel under denaturing conditions (20) in a MiniProtean apparatus (Bio-Rad) and stained with
144 Coomassie Brilliant Blue R250.

145 **Peptide fingerprinting**

146 Bands were excised from the polyacrylamide gel and subjected to in-gel tryptic digestion (40).
147 Peptides were analyzed by MALDI-TOF-MS (Matrix Assisted Laser Desorption/Ionization-Time
148 of Flight Mass Spectroscopy) using a 20mg/ml solution of 1,4-dihydroxybenzoic acid dissolved
149 in 1 part acetonitrile, 2 parts trifluoroacetic acid as the matrix. Mass spectra were collected on a
150 Bruker UltraFlex mass spectrometer (Bruker Daltonics, Bremen, Germany) that had been
151 calibrated using a peptide calibration standard (1000–4000 Da) from Bruker (part No. 206195).
152 Peptide masses were determined using Xmass (Version 5.1.5, Bruker). Proteins were identified
153 by peptide mass fingerprinting utilizing the Mascot (www.matrixscience.com) search engine.
154 Positive matches were ranked using the built-in Mowse score system of Mascot.

155 **Electron microscopy**

156 The *L. reuteri* cell pellet was prefixed in 2.0% (v/v) glutaraldehyde, 2.5% paraformaldehyde in
157 165 mM phosphate buffer, pH 7.0 for 90 min. The prefixed pellet was postfixed in 2.0% (w/v)
158 osmium tetroxide in 165 mM phosphate buffer, pH 7.2 for 120 min, followed by dehydration in
159 an ethanol series. Embedment was done in epoxy resin (Spurr, 1969). Ultrathin sections (90 nm)

160 were post-stained with 4 % (w/v) aqueous uranyl acetate and analyzed at zero-loss brightfield-

161 mode in an energy-filtered transmission electron microscope (EFTEM) (Zeiss CEM 902,

162 Oberkochen, Germany). Isolated polyhedral bodies were fixed in 1 % (v/v) glutaraldehyde and

163 after adsorption to Formvar-carbon-coated grids they were negatively stained with 2 %(w/v)

164 uranylacetate, pH 4.5. Samples were analyzed by EFTEM and images were recorded, in general,

165 with a Charge-Coupled-Device camera (Proscan Electronic Systems, Scheuring, Germany).

166 **Purification of diol dehydratase**

167 The purification procedure for diol dehydratase was carried out as described previously (Schutz

168 and Radler, 1984; Sauvageot et al., 2002). *L. reuteri* cells harvested by centrifugation at 3000 g

169 for 10 min were washed twice in K_2HPO_4 I buffer (10 mM, pH 7.2, 1 mM dithiothreitol and 1

170 mM phenylmethylsulfonyl fluoride) and then washed in 10 ml of degassed K_2HPO_4 II buffer (10

171 mM, pH 7.2 containing 5 mM dithiothreitol). Cell lysis was performed using SoniPrep 150 (MSE

172 UK Ltd) fitted with a 9 mm-diameter disrupter horn and an output of 12 microns. One mg of

173 deoxyribonuclease I was added to the lysed cells and the cell debris was removed by

174 centrifugation at two different rcf (3,000 g, 10 min and 15,500 g, 20 min).

175 The extract was homogenized with 1 volume of ammonium sulphate solution at 456 g/l to obtain

176 40 % saturation. The homogenate was incubated on ice for 1 h and centrifuged at 15,500 g for 20

177 min. The pellet containing the enzyme was resuspended in 1 ml of K_2HPO_4 II buffer and the

178 active fraction purified by gel exclusion chromatography. The enzyme preparation was loaded on

179 to a Sephacryl S300H (Sigma) column (30 x 1.5 cm) equilibrated with K_2HPO_4 II buffer.

180 Chromatography was conducted at a flow rate of 0.35 ml/min. Fractions possessing the highest

181 dehydratase activity were pooled together and stored at -70°C until further use.

182 **Diol dehydratase assay**

183 The activity of diol dehydratase was measured by the 3-methyl-2-benzothiazolinone hydrazone
184 method (53). One unit of diol dehydratase activity is defined as the amount of enzyme that
185 catalyzes the formation of 1 μmol of propionaldehyde per min per mg protein from 0.2 M 1,2-PD
186 (propanediol is used because of rapid inactivation of the enzyme over periods of more than a
187 minute by glycerol (53)). The presence of differential diol dehydratase and glycerol dehydratase
188 activity in organisms grown on different substrates was sought by establishing
189 (glycerol/propanediol)1 min, the ratio of glycerol dehydrating and 1,2-PD-dehydrating activities,
190 measured by duplicate 1-min assays using glycerol and 1,2-PD as substrates, as described by
191 Toraya (49).

192 **Acrolein (prop-2-enal) Detection** as a quantitative assay of reuterin (3-HPA) production was
193 determined by the method of Smiley and Sobolev (43), as practised by Rodriguez (32) with
194 modifications: following induction overnight in MRS-MOD broth plus glycerol (20 mM) and/or
195 1,2-PD (50 mM), cultures were standardised at OD_{600} with addition of MRS-MOD. Supernatant
196 (300 μl) from 1 ml volume of culture was incubated for one hour in MRS-MOD with glycerol
197 (200 mM) and/or 1,2-PD (50 mM) were mixed with 150 μl of tryptophan solution (3 g/1 in 0.1
198 mol/l HCl) and 600 μl of 35% HCl. The mixture was heated at 60°C for 5 min. 3-HPA (reuterin)
199 produced by bacterial metabolism was detected by dehydration to acrolein (prop-2-enal),
200 developing a yellow colour assayed at 490 nm against an acrolein standard. Bacteria-free culture
201 media were assayed as controls.

202 **Cobalamin production**

203 Cobalamin production was determined using a bioassay on sonicated cells grown in synthetic
204 vitamin B_{12} assay broth (Merck, Darmstadt, Germany) at 37°C for 3 days. Bioassay plates were
205 prepared as described previously (31) with two different indicator strains (*Salmonella enterica*

206 Serovar Typhimurium *metE cysG*, AR3612; and *Salmonella enterica* Serovar typhimurium *cbiB*
207 *metE*, AR2680) (31). AR2680 requires cobinamide or later intermediates for restoration of
208 growth, whereas AR3612 can grow in the presence of the earlier intermediate cobyric acid.

209 **1,2-Propanediol metabolism**

210 *L. reuteri* was grown in MRS-MOD medium supplemented with 50 mM 1,2-PD at 37°C in
211 anaerobic conditions for 8 days. Two ml samples were removed at different time points and
212 pelleted. Supernatant was stored at -20°C until the assays were carried out. We used a gas
213 chromatography assay following a published method (3) using a Chrompack CP-Sil 5 CB, 25 m x
214 0.25 mm with a 0.4 μm film thickness (stationary phase: 100 % dimethylpolysiloxane)
215 Measurement of 1,2-PD and its metabolites (1-propanol, propionic acid and propionaldehyde)
216 was carried out using 20 mM 1-butanol as an internal concentration standard. The temperature
217 program was set at 80°C for 2 min followed by 20°C/min temperature increase to 160°C. The
218 total time for chromatographic separation of each sample was 10 min.

219 **PCR**

220 The *pdu* operon from *L. reuteri* was amplified using primers containing *Sal*I restriction site*s*
221 (forward primer 5’-AGATGTCGACTTTCAACGGTGATGAGTGGA-3’ and reverse primer 5’-
222 AGATGTCGACTTGTGGCCATGATTTAGCAA-3’). Primers were designed with Primer3 (33)
223 based on a region of the genome of *L. reuteri* DSM 20016T (genome sequence kindly made
224 available by Gerald Tannock) identified on TBLASTX searching to be more than 70% identical
225 to the published *L. collinoides* diol dehydratase *pdu* operon (39). PCR amplification was carried
226 out with a hotstart enzyme possessing 3' to 5' proofreading activity, Platinum HiFi *Taq* DNA
227 polymerase (Invitrogen), using the program: initial denaturation at 94°C, 2 min; 30 cycles of
228 94°C, 30 s; 58°C, 30 s; 68°C, 22 min and a final elongation at 68°C, 25 min. The amplicon was

229 purified using the gel extraction kit (Qiagen) and digested with the restriction enzyme *Pst*I (New
230 England Biolabs).

231 **DNA sequence analysis**

232 Artemis (34) was used to define open reading frames in a section of *L. reuteri* DSM 20016T
233 genome sequence which was identified by BLASTP (2) similarity with *cob-pdu* operon genes
234 from *L. collinoides* (39) and GenBank. Similar segments were sought in other *Lactobacillus*
235 sequences in GenBank. A cre motif search was carried out using the program DNA-pattern at the
236 RSA-tools website http://rsat.ulb.ac.be (54) with the input string WTGNAANCGNWNNCW (25)
237 on the DNA sequence contig from *L. reuteri* DSM 20016T incorporating the *pdu* operon. The
238 *pocR-pduA* intergenic interval in the various identified *Lactobacillus sp* was examined with
239 DNA-pattern, MEME (5) and Virtual Footprint (26). Promoter prediction was performed with
240 bprom at
241 http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb.

242 **Primer extension analysis**

243 Total RNA was isolated using ToTALLY RNA™ (Ambion) from *L. reuteri* grown in MRS-
244 MOD medium supplemented with 50 mM 1,2-PD, and 50 mM 1,2-PD and 100 mM glucose,
245 respectively. Primer extension reactions were carried out as described by Ventura *et al* (55), with
246 some modifications. Briefly, around 15-20 µg of the RNA from above step was mixed with 1
247 pmol of primer (5'CAGCTTTTACCATT GCATCAGCAGC- 3') labelled with IRD800 (MWG
248 Biotech) and 2 µl of buffer H (2 M NaCl, 50 mM PIPES, pH6.4). The mixture was denatured at
249 80°C for 5 min followed by incubation for 60 min at 45°C. After addition of 18 µl of 5x First
250 standard buffer (supplied with Superscript III Reverse Transcriptase (Invitrogen)), 10 µl of 0.1 M
251 DTT, 20 µl dNTPs mix (2.5 mM each), 1 µl of 200 U/µl Superscript III Reverse Transcriptase,

252 and 41 µl of double-distilled water, the mixture was incubated at 45°C for 2 h. The product was
253 then precipitated with 250 µl of ethanol/acetone (1:1) and the pellet was washed with 80% ice
254 cold ethanol and dissolved in 4 µl of distilled water. The cDNA was separated on an 8%
255 polyacrylamide-urea gel along with the mixture from a sequencing reaction (Thermo Sequence
256 Fluorescent labelled Primer Cycle Sequencing Kit, Amersham) conducted with the same primer
257 that was used for the primer extension reaction and detected with a LiCor Sequencer machine.

258 **Sequence data**

259 The DNA sequence shown in figure 3 has been deposited in GenBank Accession EU167935.

260 **Results**

261 **Electron microscopy**

262 Ultra-thin sections of *L. reuteri* 20016 grown in the presence of 1,2-PD alone or with initial
263 glucose in addition to 1,2-PD showed the presence of polygonal intracellular bodies
264 approximately 150 nm in diameter resembling metabolosomes described from Gram-negative
265 organisms and *L. collinoides* (38) (Figure 2). From Figure 2a and d it is clear that the
266 metabolosome is covered by a single layered shell. *L. reuteri* 20016 grown in the absence of 1,2-
267 PD did not show metabolosomes inside the cells (data not shown). Metabolosome extracts
268 showed particles of similar size with evidence of surface layer disruption (Figure 2b).

269 **Diol dehydratase activity**

270 *L.reuteri* 20016 showed maximal diol dehydratase activity when incubated in 1,2-PD plus
271 glucose media (Table 1). It showed minimal diol dehydratase activity when incubated on media
272 containing glucose only, or glucose plus glycerol. *L. reuteri* 100-23 showed minimal levels of
273 diol dehydratase activity on incubation with glucose, glycerol, or 1,2-PD containing media. There
274 was no evidence of induction of a distinct glycerol dehydratase with more affinity for glycerol
275 than 1,2-propanediol by incubation with glycerol in either organism. In *Klebsiella pneumoniae*
276 expressing both glycerol dehydratase and diol dehydratase, (glycerol/propanediol)1 min
277 dehydratase activity is 2.6 in organisms pre-incubated with glycerol and 0.7 for those incubated
278 with propanediol (49).

279 **SDS-PAGE protein analysis and peptide fingerprinting**

280 Four predicted proteins from the *L. reuteri* 275 *pdu* operon were identified by MALDI-TOF
281 fingerprinting in the diol dehydratase-positive fractions of whole cell lysate of *L. reuteri* DSM
282 20016 grown on MRS medium with glucose and 1,2-propanediol (Figure 3, Table 2).

283 **Cobalamin and 3-HPA production**

284 Growth of both *Salmonella* indicator strains (*Salmonella enterica* Serovar Typhimurium *metE*
285 *cysG*, AR3612; and *Salmonella enterica* Serovar typhimurium *cbiB metE*, AR2680) was
286 promoted by cell extracts of *L. reuteri* DSM 20016, indicating the production of cobinamide or a
287 later intermediate on the route to cobalami. Beta hydroxypropionaldehyde (reuterin) production
288 from glycerol by *L. reuteri* DSM 20016 was detected by dehydration to the pigmented aldehyde
289 acrolein (prop-2-enal). Maximal production was associated with overnight induction with both
290 glycerol and 1,2-PD prior to the assay (Table 3). Addition of 1,2-PD to the glycerol substrate for
291 the assay had no inhibitory effect on reuterin production, rather increasing it 6-fold. *L. reuteri*
292 100-23 produced either no detectable reuterin, or very small amounts of reuterin at the limits of
293 detection of the assay in all conditions tested.

294 **Growth characteristics and 1, 2-propanediol metabolism**

295 *L. reuteri* 20016 grew faster to a higher OD_{600} in MRS-MOD medium with the addition of 1,2-
296 PD than in the basal MRS-MOD medium (Figure 4a), but not as rapidly as when glucose was
297 added. In contrast, *Lactobacillus reuteri* 100-23 obtained no growth advantage when 1,2-PD was
298 added to the basal medium, but showed similar growth to *L. reuteri* 20016 in glucose-containing
299 media (Figure 4a). Approximately equimolar concentrations of 1-propanol (0.53 of time zero 1,2-
300 PD molar concentration) and propionic acid (0.45 of time zero 1,2-PD molar concentration) were
301 produced by *L. reuteri* 20016 (Figure 4b) from MRS-MOD medium with 1,2-PD suggesting a
302 disproportionation reaction was taking place. No decline in propionate concentration was
303 observed in culture supernatant over 8 days, showing that propionate excreted into the culture
304 supernatant was not being taken up and further metabolised. No change in propanediol
305 concentration was observed in a bacteria-free MRS-MOD propanediol medium, and only a 3%

306 decrease in propanediol concentration was seen with incubation of *L. reuteri* 100-23 in this
307 medium over eight days (Figure 4b), showing minimal metabolism. Small amounts of
308 propionaldehyde (an intermediate in the disproportion reaction), a maximum of 2.65 mM, were
309 detected in culture supernatant of *L. reuteri* 20016 only (Figure 4b).

310 **DNA sequence analysis**

311 Genes resembling *S. enterica* Serovar Typhimurium linked *cob-pdu* operons were found in *L.*
312 *reuteri* JCM 1112/DSM 20016 (Refseq NZ AAOV00000000) (Figure 5), *L. brevis* ATCC 367
313 (Refseq CP000416)(24) and *L. hilgardii* (locus AY061969). *L. reuteri* 100-23 did not contain
314 genes resembling the *S. enterica* Serovar Typhimurium *cob-pdu* operons. A cre sequence was
315 detected in the *pocR-pduA* intergenic interval of *L. reuteri* DSM 20016
316 (TTGTAAGCGATTTCT) and *L. collinoides* (TTGAAAGCGTTTACT). MEME detected the
317 consensus motif GAAAGCGTTT when applied to the dataset of the *pduA-pocR* intergenic
318 sequences of *L. reuteri*, *L. brevis*, *L. collinoides* and *L. hilgardii*. This conforms to part of the cre
319 consensus sequence. The transcription start site of the *L. reuteri pduA* gene on induction by 1,2-
320 PD was immediately upstream of the identified cre sequence (Figure 5,6).

321 **PCR**

322 Using genome sequence data from *L. reuteri* DSM 20016 (also referred to as *L. reuteri* F275), the
323 putative *Lactobacillus reuteri pdu* operon was amplified by PCR from *L. reuteri* DSM 20016
324 (NCDO 2589), resulting in an amplicon compatible with the predicted size of 21,714 bp (Figure
325 5). *In silico* digestion of the *L. reuteri* DSM 20016 *pdu* locus with the restriction enzyme *Pst*I
326 indicated four restriction sites, which were confirmed by digesting the *L. reuteri* amplicon with
327 *Pst*I to obtain the predicted size and number of DNA fragments.

328

329 **Discussion**

330 We present the first demonstration that the antimicrobial agent-producing organism *Lactobacillus*
331 *reuteri* has the capacity to synthesize a bacterial microcompartment (carboxysome or
332 metabolosome). The organism produced a cobalamin-dependent diol dehydratase enzyme
333 induced by 1,2-propanediol, as in Gram negative bacteria containing the *pdu* operon. Linked
334 cobalamin synthesis and propanediol utilisation operons were present in the *L. reuteri DSM*
335 *20016* genome sequence, and the entire *pdu* (propanediol utilisation) operon was amplified from
336 a laboratory strain of *L. reuteri DSM 20016* by PCR, confirming its presence in the propanediol-
337 metabolising organism. Dismutation of 1,2-PD has been reported from another *Lactobacillus sp*,
338 *Lactobacillus diolivorans*, an *L. buchneri*-like organism from maize sileage (19). However, no
339 assay of cobalamin production was reported and metabolosomes were not seen on electron
340 microscopy of *L. diolivorans* growing on media incorporating 1,2-PD.
341
342 The conversion of 1,2-PD to propanol and propionate with the transient presence of
343 propionaldehyde we have observed (Figure 4b) suggests a pathway as described for 1,2-
344 propanediol utilisation in *Salmonella* (8, 22, 36) (Figure 7). Genes specifying all the enzymes
345 required shown in Figure 7 were present in the *L. reuteri* F275 (DSM 20016) *pdu* operon (Figure
346 5).
347
348 However, in *Enterobacteriaceae* like *Salmonella* capable of 1,2-PD utilisation via a
349 metabolosome-associated diol dehydratase there are significant further onward metabolic
350 connections for the dismutation products which are not present in *Lactobacilli.* In *Salmonella,* the
351 propionate product of 1,2-PD utilisation can be coupled via the methylcitrate cycle to aerobic
352 respiration (15, 28) or tetrathionate reduction (30), allowing growth on 1,2-PD as a sole carbon

353 and energy source. In the absence of oxygen or tetrathionate, *Salmonella sp* can only grow on
354 defined no-carbon media containing added 1,2-propanediol to which yeast extract has also been
355 added (30). It is proposed that this represents fermentative growth using a carbon source in the
356 yeast extract with energy from propanediol dismutation (30). The pathways by which *Salmonella*
357 sp utilise propionate have not been reported from *Lactobacillus sp*, and no evidence for them is
358 apparent from the *L. reuteri* genome sequence. We observed a steady increase in propionate
359 levels in culture supernatant from *L. reuteri* 20016 grown in MRS media with 1,2-PD over eight
360 days of continuous culture, suggesting propionate is excreted and cannot be utilised by the
361 organism. Slower growth rates were seen in MRS-MOD medium when 1,2-PD alone was added
362 to the basal medium compared to glucose, but there was an advantage compared with the basal
363 MRS-MOD medium (Figure 3). It is likely that *L. reuteri* growth on MRS-MOD medium with
364 1,2-PD is, as in non-respiring *Salmonella*, a result of fermentation of other carbon sources such as
365 yeast extract in the complex MRS media combined with energy from 1,2-PD dismutation (Figure
366 7). The control organism *L. reuteri* 100-23 (in the genome sequence of which no *pdu* operon is
367 apparent) gained no growth advantage from the addition of 1,2-PD to the basal medium, and was
368 only able to metabolise a small amount of 1,2-PD over a period of eight days (Figure 3).
369

370 The enzyme specified by the *pdu* operon *pduCDE* genes, diol dehydratase, is responsible for
371 conversion of 1,2-propanediol to the intermediate propionaldehyde. Interestingly, the enzyme
372 responsible for glycerol conversion to 3-HPA in *L. reuteri* (Figure 1) has previously been
373 described as a glycerol dehydratase (47), but is capable of acting as a propanediol dehydratase
374 (47). The presence of two isofunctional related enzymes, (glycerol and propanediol dehydratase)
375 in *L. reuteri*, as in *Klebsiella pneumoniae,* was inferred from the existence of two peaks of

376 propanediol dehydratase activity on cell extracts separated by DEAE-cellulose chromatography
377 (47). This left the possibility that reuterin production could be dependent on either one of two
378 isofunctional enzymes. The *L. reuteri* F275 (DSM 20016) genome sequence has recently been
379 circularised (http://genome.jgi-psf.org/finished_microbes/lacre/lacre.info.html) and BLAST
380 searching does not reveal a distinct glycerol dehydratase in addition to the diol dehydratase
381 linked with cobalamin synthesis. That is, the only candidate enzyme identifiable from the
382 genome sequence for production of 3-HPA from glycerol forming the antimicrobial reuterin (56)
383 is the metabolosome-associated propanediol-induced diol dehydratase we describe. Supporting
384 this, we found no phenotypic evidence of a distinct glycerol-induced dehydratase in *L. reuteri*
385 20016 (Table 1), and maximal reuterin production by *L. reuteri* 20016 was associated with pre-
386 incubation with 1,2-PD in addition to glycerol (Table 2). Very small amounts of reuterin were
387 produced in the absence of 1,2-PD in pre-incubation or assay conditions (Table 2). The *L. reuteri*
388 100-23 strain lacking the metabolosome-associated diol dehydratase in its unpublished genome
389 sequence was unable to synthesize more than trace amounts of reuterin (at most, less than 6% of
390 that detected from *L. reuteri* 20016) (Table 2) and had very low levels of diol dehydratase
391 activity, irrespective of substrate induction (Table 1).
392

393 While individual metabolosomes resembled electron microscopy reports from *Salmonella* (8, 14,
394 41), fewer metabolosomes were observed in each bacterial cell and metabolosomes were
395 agglomerated (Figure 2a,c). Similar electron microscopy appearances have been reported from
396 *Lactobacillus collinoides* (38), which also expresses a metabolosome-associated diol dehydratase
397 (39) but does not synthesize cobalamin. Biochemical data supported these qualitative EM
398 appearances, showing a reduced specific enzyme activity compared with Gram-negative

399 organisms: maximal diol dehydratase activity per mg of whole cell extract was comparable with
400 that reported from *L. collinoides* (39) and approximately a quarter of that reported from
401 *Salmonella* (14).
402
403 Although the *pdu* operon is substantially similar in gene number and order in *Salmonella* and
404 *Lactobacillus reuteri,* DNA sequence analysis upstream of the *pdu* operon suggests it may be
405 regulated differently (Figure 4). The linked *cob/pdu* metabolosome operons in a Gram negative
406 background are regulated by Crp and Arc (1). In *Lactobacillus sp* as for other Gram-positive
407 organisms (35), catabolite repression generally occurs via HPr [HPr(Ser-P)], the small
408 phosphocarrier protein of the phosphoenolpyruvate-sugar phosphotransferase system, and CcpA
409 protein (17) (6), operating via short catabolite responsive elements (cre) in DNA sequence (4, 17,
410 25). Although 1,2-PDl utilisation operons have been described for other *Lactobacillus* species,
411 cre elements have not previously been noted in connection with them. We identified a cre
412 consensus sequence in the *L. reuteri pdu* operon upstream of *pduA*, the first gene in the *pdu*
413 operon. We found complete or partial cre sequences upstream of *pduA* in all other available DNA
414 sequences from *Lactobacillus sp* containing this operon. In *L. reuteri*, the centre of the *cre*
415 element is +17 base pairs downstream of the transcription start site of the initial gene in the *pdu*
416 operon when induced by 1,2-PD, and +22 base pairs relative to the end of the putative -10
417 sequence (Figure 5). In *Lactococcus lactis*, a cre element in this orientation is associated with
418 strong CcpA-dependent repression (59).
419
420 The requirement of a complex 22-gene 1,2-propanediol utilisation operon for this apparently
421 simple process has been attributed to the need to contain the intermediate compound
422 propionaldehyde within a protein compartment or metabolosome, either to reduce toxicity (36) or

423 to prevent its loss as a gas by the cell (29). As reported from metabolosome-containing *S.*
424 *enterica* metabolising 1,2-PD (36), we detected only small amounts of propionaldehyde in culture
425 supernatant of 1,2-PD-metabolising *L. reuteri* (Figure 3b), suggesting retention within the
426 metabolosome. It has been suggested that in the metabolosome associated with the ethanolamine
427 utilisation operon in *S. enterica*, that the mechanism of aldehyde retention is based on reduced
428 loss of the aldehyde intermediate (in this case acetaldehyde) by evaporation, possibly by creating
429 a low pH within the compartment, rendering aldehydes more likely to convert to a less volatile
430 acetal (29). However, in the *S. enterica* 1,2-PD utilisation metabolosome, assays of *pduA* deletion
431 mutants not producing the metabolosome shell but retaining metabolic activity, showed that
432 increased propionaldehyde evaporation was not a major factor affecting 1,2-PD metabolism (36).
433 If, as we suggest, the metabolosome-associated diol dehydratase is also responsible for reuterin
434 (3-HPA) production from glycerol, then the fact this aldehyde is excreted by the organism,
435 suggests that either 3-HPA is not produced within the aldehyde-retaining metabolosome (i.e. a
436 significant amount of diol dehydratase is outside the metabolosome in the cytoplasm, unlike the
437 situation in *Salmonella* (8)), or alternatively, the NAD-dependent oxidoreductase which removes
438 3-HPA in *L. reuteri* by conversion to 1,3-propanediol (Figure 1) might not be localised in the
439 metabolosome in the same way that PduP CoA-acylating propionaldehyde dehydrogenase is
440 present within the 1,2-propanediol-metabolising metabolosome (22) (Figure 7). That is, effective
441 aldehyde retention by the metabolosome requires the presence of specific aldehyde-metabolising
442 enzymes within the metabolosome.
443
444 Carboxysomes in cyanobacteria affect internal cytoplasmic pH (7) and concentrate protons.
445 There is recent evidence for regulation of the *pdu* operon by external pH in *L. reuteri*. During

446 revision of this manuscript it was reported that gene transcription assays using a DNA microarray
447 based on partial genome sequence data from *L. reuteri* ATCC 55730 showed eleven genes from
448 the *pdu* operon were downregulated by dilution and incubation at pH5.1 compared with at pH 2.7
449 (57). Lactobacilli including *L. reuteri* are heterotrophic fermentative organisms that obtain energy
450 by substrate level phosphorylation and require high levels of different nutrients to maintain a
451 sufficient proton motive force for viability (18). While neutrophilic bacteria like *E.coli* respond to
452 changes in external pH (pHe) by maintaining a relatively constant internal pH (pHi) at the
453 expense of a large proton gradient across the cell wall, fermentative Lactic acid bacteria decrease
454 pHi in response to decreasing pHe, to maintain a constant transmembrane proton gradient (9, 42).
455 Proton concentration within *Lactobacillus* sp metabolosomes could potentially raise pHi of the
456 remaining cytoplasm, compromising efforts to maintain a constant transmembrane proton
457 gradient in acidified growth media.
458
459 However, we have shown that the metabolosome-associated propanediol utilisation operon is 1,2-
460 PD-induced as in Gram-negative organisms, and functions in a *Lactobacillus* intracellular
461 background, despite differences in pH homeostasis from organisms where it has been mainly
462 studied to date. This finding reinforces the evidence (21) that this very large and complex
463 metabolic operon is nevertheless frequently horizontally transmitted between different bacteria.
464 Further study of the constraints of operating in a fermentative background will shed new light on
465 the electrochemical properties of the metabolosome.
466

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651

652 **Figure Legends**

653 Figure 1

654 Title: Beta-hydroxypropionaldehyde (3-HPA) production and metabolism in *L. reuteri*

655

656 Figure 2

657 Title: *L. reuteri* 20016 produces metabolosomes

658 Legend

659 a. cell section electron micrograph after growth in MRS broth supplemented with 65 mM 1, 2-

660 propanediol and 15 mM glucose at 37°C for 18 h

661 b. (inset) extracted metabolosome from cells grown as in a).

662 c. *L. reuteri* grown on MRS broth supplemented with 65 mM 1, 2-propanediol at 37°C for 18 h

663 d. (inset) enlarged view of metabolosomes shown in c.

664 Arrow in a and c indicates metabolosomes

665

666 Figure 3

667 Title: SDS-PAGE separation of *L. reuteri* total cell protein fractions and MALDI-TOF-identified

668 proteins

669 Legend

670 Lane 1 protein molecular weight marker

671 Lane 2 diol dehydratase positive fraction 1

672 Lane 3 diol dehydratase positive fraction 2 (immediately below fraction 1 in sucrose density

673 gradient)

674 *L. reuteri* grown in MRS broth supplemented with 15 mM glucose and 50 mM 1, 2-propanediol
675 at 37°C for 36 h
676
677 Figure 4
678 Title: Growth characteristics of *L.reuteri* strains and anaerobic propanediol metabolism
679 Legend
680 a. Growth curves of *L.reuteri* 20016 and *L.reuteri* 100-23. X axis: time post inoculation. Y
681 axis: optical density readings at 600 nm
682 ▲ solid line: *L.reuteri* 20016 in MRS-MOD with 50 mM glucose.
683 ∆ dotted line: *L.reuteri* 20016 in MRS-MOD with 50 mM 1,2 propanediol.
684 ▲ dashed line: *L.reuteri* 20016 in unsupplemented MRS-MOD.
685 ○ dashed line *L.reuteri* 100-23 in MRS-MOD with 50 mM glucose.
686 ○ solid line: *L.reuteri* 100-23 in MRS-MOD with 50 mM 1,2 propanediol.
687 b. Propanediol metabolism by *L.reuteri* 20016 or *L.reuteri* 100-23 in MRS-MOD with 1,2
688 propanediol. X axis time post inoculation. Y axis: metabolite concentration in mM.
689 ■ dashed line: propanediol concentration in bacteria-free control
690 ○ solid line: *L. reuteri* 100-23, propanediol concentration
691 □ solid line: *L. reuteri* 20016, propanediol concentration
692 ▼ dashed line: *L. reuteri* 20016, propanol concentration
693 • dotted line: *L. reuteri* 20016, propionate concentration
694 ♦ dashed line *L. reuteri* 20016, propionaldehyde concentration
695
696 Figure 5

697 Title: The *pdu* operon of *L. reuteri*

698 Legend

699 Predicted open reading frames gene assignment by comparison with *S. enterica* Serovar
700 Typhimurium (nomenclature of labelled cobalamin synthesis genes follows *Salmonella*
701 convention). Consensus cre sequence is boxed, predicted -35 and -10 promoter sequences and
702 ribosomal binding site underlined, start codon of *pduA* in bold. Transcriptional start site when
703 induced by propanediol is indicated by letter in larger font. Extent of PCR product and predicted
704 restriction sites shown below.

705

706 Figure 6:

707 Title: *pduA* gene transcription start site on propanediol induction

708 Legend

709 Primer extension products were obtained by using total RNA extracted from *L. reuteri* grown on
710 MRS-MOD medium supplemented with 50 mM 1,2-propanediol (lane 1) and 50 mM 1,2-
711 propanediol+ 100 mM glucose (lane 2), respectively. Start point of transcription is boxed.

712

713 Figure 7

714 Title: Proposed pathway of cobalamin-dependent 1,2-propanediol metabolism in *L. reuteri*

715 Legend

716 Metabolic endpoints underlined. *Metabolic intermediates retained within the metabolosome.

717

TABLE 1. *L. reuteri* diol dehydratase activity after 36 hours incubation with different substrates

Growth substrate*	Diol dehydratase activity units/mg (measured with 1,2 propanediol substrate)		(Glycerol/propanediol)1 min dehydratase activity	
	L. reuteri 20016	*L. reuteri* 100-23	*L. reuteri* 20016	*L. reuteri* 100-23
Glucose 15 mM	0.04	0.04	0.60	0.91
Glucose 15 mM + 1,2-PD 50 mM	0.55	0.04	0.91	0.91
Glucose 15 mM + glycerol 50 mM	0.07	0.04	0.89	0.96

*Carbon sources added to MRS-MOD (*L. reuteri* 100-23 requires glucose in addition to 1,2-PD to grow in MRS-MOD). 1 unit of diol dehydratase activity is defined as the amount of enzyme activity catalyzing the formation of 1 μmol propionaldehyde. (Glycerol/propanediol)1 min activity represents the ratio of dehydrating assay activity detected when glycerol is the assay substrate compared with l,2-propanediol as substrate, measured by 1-minute assays.

TABLE 2. Peptide mass fingerprinting of metabolosome components

Predicted Molecular Weight	Identity assigned in *Lactobacillus reuteri* F275 genome (locus tag)	NCBI accession no.	Mascot search result		
			No. of peptides matched	Mowse Score (probability that the observed match is a random event)	% sequence coverage
62566	Glycerol dehydratase large subunit PduC (Lreu 1747)	gi|148544953	19	197 (1.2e-13)	49
25849	Propanediol dehydratase, medium subunit PduD (Lreu 1746)	gi|148544952	8	131 (5e-07)	55
23947	Propanediol utilization protein PduL (Lreu 1740)	gi|148544946	11	99 (0.00079)	67
17007	Protein of unknown function DUF336/PduObis (Lreu 1736)	gi|148544942	5	85 (0.02)	63

Mascot search was used to compare the MALDI-TOF MS data obtained for sample proteins to predicted spectra for proteins present in the NCBI database. Protein scores greater than 80 are significant ($p<0.05$). ND = not determined.

TABLE 3. Beta hydroxypropionaldehyde (reuterin) production from glycerol and/or propanediol in one hour by *L. reuteri* strains induced with glycerol, or glycerol and 1,2 propanediol

Overnight induction conditions in MRS-MOD	Beta hydroxypropionaldehyde (3-HPA) assay substrate	Beta hydroxypropionaldehyde (3-HPA) produced (mM)*	
		L. reuteri 20016	*L. reuteri* 100-23
Glycerol	Glycerol	0.08	0.00
	Glycerol +1,2-PD	0.08	0.00
	1,2-PD	0.00	0.03
Glycerol +1,2-PD	Glycerol	0.46	0.03
	Glycerol +1,2-PD	0.52	0.03
	1,2-PD	0.08	0.03

1,2-PD (1,2 propanediol) in all cases at 50 mM, glycerol induction 20 mM, glycerol 200 mM for 3-HPA production assay conditions
*(measured by dehydration to acrolein/prop-2-enal)