Lactobacillus reuteri DSM 20016 Produces Cobalamin-Dependent Diol Dehydratase in Metabolosomes and Metabolizes 1,2-Propanediol by Disproportionation

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

Lactobacillus reuteri is a probiotic bacterium able to colonize the gastrointestinal tract of a wide variety of mammals and birds (12). It produces an antimicrobial agent (45) (reuterin) by fermentation of glycerol. Lactobacillus spp. (including L. reuteri) cannot grow on glycerol as a sole carbon source, but L. reuteri can use beta-hydroxypropionaldehyde (3-HPA), which it derives from glycerol, as a hydrogen acceptor in fermentation of other carbohydrates, including glucose and lactose (44). Unlike most lactobacilli, L. reuteri grown in this way on glycerol and another carbohydrate excretes large amounts of reuterin, consisting of an equilibrium mixture of different monomeric and dimeric forms of 3-HPA, which has been shown to correspond to reuterin (46, 56). There is an optimal ratio of glycerol and glucose for maximal 3-HPA production, and if excess glucose is supplied, 3-HPA is further reduced to 1,3-propanediol by a 1,3-propanediol:NAD oxidoreductase (23) (Fig. 1).

It has been suggested there are two distinct cobalamin-dependent dehydratases in L. reuteri that can produce HPA from glycerol (47). Certainly, in Klebsiella pneumoniae (49) and a variety of other Enterobacteriaceae (13, 51), two different isofunctional cobalamin-dependent enzymes, glycerol dehydratase (EC 4.2.1.30) and diol dehydratase (EC 4.2.1.28), can catalyze the key reaction of glycerol dehydratation to 3-HPA (Fig. 1) (13, 52). They can also convert a different substrate, 1,2-propanediol (1,2-PD), to propionaldehyde. Diol dehydratase genes are associated in many Enterobacteriaceae with a functional cobalamin synthesis pathway (21) and the production of a proteinaceous cellular microcompartment localizing the active enzyme, resembling the carboxysome containing ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in autotrophic bacteria (10). This structure in heterotrophic Enterobacteriaceae has been termed an enterosome (11) or a carboxysome (29) (based on a hypothesis that carbon dioxide fixation may also occur in these heterotrophic bacteria). Generic terms such as bacterial microcompartment (16) and 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 utilization operon specifies enzymes for a dismutation that converts 1,2-PD (via propionaldehyde) to approximately equal amounts of n-propanol (reduced) and propionate (oxidized). ATP is produced via substrate-level phosphorylation: 2 (CH3-CH2-COOH) + ADP + P → CH3-C2H5-COOH + CH3-CH2-COOH + ATP + 2H2O.

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. No demonstration of 1,2-PD utilization or bacterial microcompartment production in *L. reuteri* strains has previously been reported. We show that in *L. reuteri* 20016 (the type strain, originally isolated from human feces, a bacterial microcompartment is present, and inducible 1,2-PD utilization occurs, 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 utilization operons as in gram-negative bacteria, with a distinct gram-positive CRE potentially regulating gene transcription in a *Lactobacillus* background.

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** *L. reuteri* NCDO 2589 was obtained from the National Collection of Dairy Organisms, Reading, United Kingdom (now NCIMB Ltd, Aberdeen, United Kingdom). This is also known as DSM 20016, the type strain, and F275, which has recently been sequenced, and was originally isolated from human feces. *L. reuteri* 100-23 was obtained from Gerrard Tannock (University of Otago, Dunedin, New Zealand). This strain is also known as DSM 17509 and has been sequenced. It was originally isolated from the digestive tract of a rat (58). *L. reuteri* 100-23 was employed only as a negative control in the propanediol metabolism assay and growth curves. All other references to *L. reuteri* here refer to *L. reuteri* DSM 20016. *L. reuteri* strains were grown in de Man-Rogosa-Sharp (MRS) broth overnight (13a) containing 15 mM glucose at 37°C without shaking. To test for reuterin production by acrolein-based quantification, Lactobacillus strains grown in MRS broth containing 65 mM H2PO4, 0.6 g CH3COONa, 0.3 g MgSO4 were grown in conical flasks containing MRS broth supplemented with 50 mM 1,2-PD and 15 mM glucose at 37°C for 36 h. For the detection of metabolosomes using transmission electron microscopy, *L. reuteri* was grown in MRS broth containing 65 mM 1,2-PD with and without 15 mM glucose at 37°C for 18 h. For dismutation of 1,2-PD, *L. reuteri* was grown in modified MRS (MRS-MOD) medium, pH 5.7 (19), supplemented with 40 mM 1,2-PD without glucose, at 37°C under anaerobic conditions for 8 days. MRS-MOD is a complex medium containing (per liter) 5 g Bacto-peptone, 4 g Lab-Lemco (Oxoid), 2 g yeast extract, 0.5 ml Tween 80, 1.0 g K2HPO4, 3.0 g NaH2PO4, 5.0 g CH3COONa, 0.3 g MgSO4·7H2O, and 0.04 g MnSO4·H2O.

**Isolation of metabolosomes and protein separation.** Protein preparations were initially made by a modification of a published procedure (14). Briefly, *L. reuteri* grown in MRS broth containing 1,2-PD and glucose was harvested by centrifugation at 4°C, 3,000 × g for 10 min. The pellet was washed with 300 ml lysyox buffer (50 mM Tris-Cl, 0.6 M sucrose, 5 mM EDTA, 0.2% 1,2-PD [pH 8.0]), resuspended in 30 ml of the same buffer containing 5 mg/ml lysozyme, and incubated at 37°C for 2 h with occasional agitation. All further steps were scaled out at 4°C. Lysozyme-treated cells were pelleted by centrifugation at 7,500 × g for 15 min, washed with lysyox buffer, and resuspended in sonication buffer (50 mM Tris-Cl, 2 mM EDTA, 0.2% 1,2-PD [pH 8.0]) at approximately 0.1 g wet cell mass per ml. Cells were lysed by sonication, four 120-s bursts with 1-min cooling intervals on ice, using SoniPrep 150 (MSE UK Ltd). The crude cell extract obtained by sonication was mixed with an equal volume of BPER-II (Pierce, Rockford, IL) supplemented with 400 mM NaCl and 20 mM MgCl2 and incubated for 30 min at 4°C with shaking. Unlysed cells were removed by centrifugation at 12,000 × g for 10 min. The resulting supernatant was subjected to ultracentrifugation (Beckman SW-41 Ti rotor) at 49,000 × g for 90 min. The crude protein pellet was resuspended in 5 ml of TEM buffer (50 mM Tris-Cl, 1 mM EDTA, 10 mM MgCl2, 0.2% 1,2-PD [pH 8.0]) and clarified by centrifugation at 12,000 × g for 10 min. The clarified preparation was layered onto four 11-ml 35%-to-65% (wt/vol) sucrose density gradients and centrifuged at 30,000 × g for 16 h. Fractions including the pellet were taken and assayed for diol dehydratase activity. Dehydratase-positive fractions were retained in sucrose buffer, and the pellet was resuspended in 1 ml of TEM buffer and clarified by centrifugation before electron microscopy. Protein preparations for peptide fingerprinting were made by cell sonication as described above (omitting lysozyme and admixture with BPER-II), with subsequent fractionation of the total crude cell lysate by sucrose density gradient centrifugation, selecting diol dehydratase-positive fractions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation.

**Protein separation.** Aliquots (50 µg) of extracted protein were separated by SDS-PAGE using a 12.5% polyacrylamide gel under denaturing conditions (20) in a MiniProtein apparatus (Bio-Rad) and stained with Coomassie brilliant blue R250.

**Peptide fingerprinting.** Bands were excised from the polyacrylamide gel and subjected to in-gel trypptic digestion (40). Peptides were analyzed by MALDI-TOF-MS (matrix-assisted laser desorption/ionization-time of mass spectrometry) using a 20-µg/ml solution of 1,4-dihydroxybenzene (37) dissolved in 1% trifluoroacetic acid in 80% acetonitrile as the matrix. Mass spectra were collected on a Bruker UltraFlex mass spectrometer (Bruker Daltonics, Bremen, Germany) that had been calibrated with a peptide calibration standard (1,000 to 4,000 Da) from Bruker (part 206195). Peptide masses were determined using Xmass (version 5.1.5; Bruker). Proteins were identified by peptide mass fingerprinting utilizing the Mascot search engine. Positive matches were ranked using the built-in Mwose sequence system of Mascot (Matrix Science, Boston, MA).

**Electron microscopy.** The *L. reuteri* cell pellet was precipitated in 20% (vol/vol) glutaraldehyde, 2.5% paraformaldehyde in 165 mM phosphate buffer, pH 7.0, for 90 min. The prefixed pellet was postfixed in 2.0% (wt/vol) osmium tetroxide in 165 mM phosphate buffer, pH 7.2, for 120 min, followed by dehydration in an ethanol series. Embedment was done in epoxy resin (43a). Ultrathin sections (90 nm) were poststained with 4% (wt/vol) aqueous uranyl acetate and analyzed in zero-loss bright-field mode in an energy-filtered transmission electron microscope (Zeiss CEM 902, Zeiss, Oberkochen, Germany). Isolated polyhedral bodies were fixed in 1% (vol/vol) glutaraldehyde, and after adsorption to Formvar-carbon-coated grids they were negatively stained with 2% (wt/vol) uranylacetate, pH 4.5. Samples were analyzed with an energy-filtered transmission electron microscope, and images were recorded, in general, with a charge-coupled-device camera (Pросcan Electronic Systems, Scheuring, Germany).

**Purification of diol dehydratase.** The purification procedure for diol dehydratase was carried out as described previously (35a, 39a). *L. reuteri* cells harvested by centrifugation at 3,000 × g for 10 min were washed twice in K2HPO4 buffer I (10 mM, pH 7.2, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and then washed in 10 ml of degassed K2HPO4 buffer II (10 mM, pH 7.2 containing 5 mM dithiothreitol). Cell lysis was performed using SoniPrep 150 (MSE UK Ltd) fitted with a 9-mm-diameter disruptor horn and an output of 12 µm. One milligram of DNAse I was added to the lysed cells, and the cell debris was removed by centrifugation at two different reactive centrifugal forces (3,000 × g for 10 min and 15,500 × g for 20 min).

The extract was homogenized with 1 volume of ammonium sulfate solution at 456 g/liter to obtain 40% saturation. The homogenate was incubated on ice for 1 h and centrifuged at 15,500 × g for 20 min. The pellet containing the enzyme was resuspended in 1 ml of K2HPO4 buffer II, and the active fraction was purified by gel exclusion chromatography. The enzyme preparation was loaded onto a Sephacryl S300H (Sigma) column (30 by 1.5 cm) equilibrated with K2HPO4 buffer II. Chromatography was conducted at a flow rate of 0.35 ml/min. Fractions possessing the highest dehydratase activity were pooled and stored at −70°C until further use.

**Diol dehydratase assay.** The activity of diol dehydratase was measured by the 3-methyl-2-benzothiazolinone hydrazide method (53). One unit of diol dehydratase activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of propanediol per min per mg protein from 0.2 M 1,2-PD (propanediol is used because of rapid inactivation of the enzyme over periods of more than a minute by glycerol [53]). The presence of differential diol dehydratase and glycerol dehydratase activities in organisms grown on different sub-
strates was sought by establishing the ratio of glycerol dehydration and 1,2-PD dehydration activities, measured by duplicate 1-min assays using glycerol and 1,2-PD as substrates (glycerol/propanediol)\(^1\) \(100\) as described by Toraya and Fukui (49).

**Acrolein (prop-2-enal) detection.** Acrolein (prop-2-enal) detection as a quantitative assay of reuterin (3-HPA) production was carried out by the method of Smiley and Sobolev (43), as practiced by Rodriguez et al. (32) with modifications. Following induction overnight in MRS-MOD broth plus glycerol (20 mM) and/or 1,2-PD (50 mM), cultures were standardized at the same optical density (600 nm) with the addition of MRS-MOD. Supernatants (300 \(\mu\)l) from 1 ml of culture incubated for 1 h in MRS-MOD with glycerol (200 mM) and/or 1,2-PD (50 mM) were mixed with 150 \(\mu\)l of tryptophan solution (3 g/l in 0.1 mol/l HCl) and 600 \(\mu\)l of 35% HCl. The mixture was heated at 60°C for 5 min. 3-HPA (reuterin) produced by bacterial metabolism was detected by dehydration to acrolein (prop-2-enal), developing a yellow color assayed at 490 nm against an acrolein standard. Bacterium-free culture media were assayed as controls.

**Cobalamin production.** Cobalamin production was determined using a biosay on sonicated cells grown in synthetic vitamin B\(_12\) assay broth (Merck, Darmstadt, Germany) at 37°C for 3 days. Biosassay plates were prepared as described previously (31) with two different indicator strains (Salmonella enterica serovar Typhimurium metE csGl, AR3612, and S. enterica serovar Typhimurium cbiE metE, AR2680) (31). AR2680 requires cobinamide or later intermediates for restoration of growth, whereas AR3612 can grow in the presence of the earlier intermediate coobyclic acid.

**1,2-PD metabolism.** *L. reuteri* was grown in MRS-MOD medium supplemented with 50 mM 1,2-PD at 37°C under anaerobic conditions for 8 days. Two-milliliter samples were removed at different time points and pelleted. Supernatants were stored at 20°C until the assays were carried out. We used a gas chromatography assay following a published method (3) using a Chrompack CP-Sil 5 CB column, 25 m of 35% HCl. The mixture was heated at 60°C for 2 min followed by a 20°C/min temperature increase to 160°C. The total time for gas chromatographic separation of each sample was 10 min.

**PCR.** The pdu operon from *L. reuteri* was amplified using primers containing SSL restriction sites (forward primer, 5'-AGATGGTAGCTTCAACAGGTGAT GAGTTGA-3' and reverse primer, 5'-AGATGGTAGCTTCAACAGGTGAT GAGTTGA-3'). Primers were designed with Primer3 (33) based on a region of the genome of *L. reuteri* DSM 20016\(^6\) (genomic sequence kindly made available by Gerald Tannock) determined by TBLASTX searching to be more than 70% identical to the published *Lactobacillus collinoides* pdu operon. PCR amplification was carried out with a hot-start enzyme possessing 3'-to-5' proofreading activity, Platinum HiFi (Invitrogen), using the following program: initial denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 22 min; and a final elongation at 68°C for 25 min. The amplicon was purified with a gel extraction kit (Qiagen) and sequenced. The DNA sequence analysis. Artemis (34) was used to define open reading frames (ORFs) and protein translations. Briefly, around 15 to 20 \(\times\) 10^5 \(\mu\)l of 200 U/l Superscript III reverse transcriptase, and 41 \(\mu\)l of distilled water. The cDNA was separated on an 8% polyacrylamide-urea gel along with the mixture from a sequencing reaction (Thermo Sequence fluorescently labeled primer cycle sequencing kit; Amersham) conducted with the same primer that was used for the primer extension reaction and detected with a LiCor sequencer machine.

**Nucleotide sequence accession number.** The DNA sequence shown in Fig. 3 has been deposited in GenBank with accession no. EU167935.

**RESULTS**

**Electron microscopy.** Ultrathin sections of *L. reuteri* 20016 grown in the presence of 1,2-PD alone or with initial glucose in addition to 1,2-PD showed the presence of polygonal intracellular bodies approximately 150 nm in diameter resembling the metabolosomes described for gram-negative organisms and *L. collinoides* (38) (Fig. 2). From Fig. 2a and c it is clear that the metabolosome is covered by a single-layer shell. *L. reuteri* 20016 grown in the absence of 1,2-PD did not show metabolosomes inside the cells (data not shown). Metabolosome extracts showed particles of similar size with evidence of surface layer disruption (Fig. 2b).

**Diol dehydratase activity.** *L. reuteri* 20016 showed maximal diol dehydratase activity when incubated in medium containing 1,2-PD plus glucose (Table 1). It showed minimal diol dehydratase activity when incubated in medium containing glucose only or glucose plus glycerol. *L. reuteri* 100-23 showed minimal levels of diol dehydratase activity on incubation with glucose\(_-\), glycerol\(_-\), or 1,2-PD-containing medium. There was no evidence of induction of a distinct glycerol dehydratase with more affinity for glycerol than 1,2-PD by incubation with glycerol in either organism. In *K. pneumoniae* expressing both glycerol dehydratase and diol dehydratase, the (glycerol/propanediol)\(^1\) min dehydratase activity is 2.6 in organisms preincubated with glycerol and 0.7 in those incubated with propanediol (49).

**SDS-PAGE protein analysis and peptide fingerprinting.** Four predicted proteins from the *L. reuteri* 275 pdu operon were identified by MALDI-TOF fingerprinting in the diol dehydratase-positive fractions of whole-cell lysate of *L. reuteri* DSM 20016 grown on MRS medium with glucose and 1,2-PD (Fig. 3; Table 2).

**Cobalamin and 3-HPA production.** Growth of both *Salmonella* indicator strains (AR3612 and AR2680) was promoted by cell extracts of *L. reuteri* DSM 20016, indicating the production of cobinamide or a later intermediate on the route to cobalamin. 3-HPA (reuterin) production from glycerol by *L. reuteri* DSM 20016 was detected by dehydration to the pigmented aldehyde acrolein (prop-2-enal). Maximal production was associated with overnight induction with both glycerol and 1,2-PD prior to the assay (Table 3). Addition of 1,2-PD to the glycerol substrate for the assay had no inhibitory effect on reuterin production but rather increased it sixfold. *L. reuteri* 100-23 produced either no detectable reuterin or very small amounts of reuterin at the limits of detection of the assay under all conditions tested.

**Growth characteristics and 1,2-PD metabolism.** *L. reuteri* 20016 grew faster to a higher optical density at 600 nm in MRS-MOD medium with the addition of 1,2-PD than in the basal MRS-MOD medium (Fig. 4a) but not as rapidly as when glucose was added. In contrast, *L. reuteri* 100-23 obtained no growth advantage when 1,2-PD was added to the basal medium.
but showed growth similar to that of *L. reuteri* 20016 in glucose-containing medium (Fig. 4a). Approximately equimolar concentrations of 1-propanol (53% of time zero 1,2-PD molar concentration) and propionic acid (45% of time zero 1,2-PD molar concentration) were produced by *L. reuteri* 20016 (Fig. 4b) from MRS-MOD medium with 1,2-PD, suggesting that a disproportionation reaction was taking place. No decline in propionate concentration was observed in culture supernatant over 8 days, showing that propionate excreted into the culture supernatant was not being taken up and further metabolized. No change in propanediol concentration was observed in a bacterium-free MRS-MOD propanediol medium, and only a 3% decrease in propanediol concentration was seen with incubation of *L. reuteri* 100-23 in this medium over 8 days (Fig. 4b), showing minimal metabolism. Small amounts of propionaldehyde (an intermediate in the disproportion reaction), a maximum of 2.65 mM, were detected in culture supernatant of *L. reuteri* 20016 only (Fig. 4b).

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

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Diol dehydratase activity (U/mg) (measured with 1,2-propanediol substrate)</th>
<th>(Glycerol/propanediol) dehydratase activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th><em>L. reuteri</em> 20016</th>
<th><em>L. reuteri</em> 100-23</th>
<th><em>L. reuteri</em> 20016</th>
<th><em>L. reuteri</em> 100-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (15 mM)</td>
<td>0.04</td>
<td>0.04</td>
<td>0.60</td>
<td>0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (15 mM) + 1,2-PD (50 mM)</td>
<td>0.55</td>
<td>0.04</td>
<td>0.91</td>
<td>0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (15 mM) + glycerol (50 mM)</td>
<td>0.07</td>
<td>0.04</td>
<td>0.89</td>
<td>0.96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Carbon source added to MRS-MOD (*L. reuteri* 100-23 requires glucose in addition to 1,2-PD to grow in MRS-MOD).

<sup>b</sup> One unit of diol dehydratase activity is defined as the amount of enzyme activity catalyzing the formation of 1 μmol propionaldehyde.

<sup>c</sup> Ratio of dehydrating assay activity detected when glycerol is the assay substrate to that with 1,2-propanediol as the substrate, measured by 1-min assays.

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**FIG. 2.** *L. reuteri* 20016 produces metabolosomes. (a) Cell section electron micrograph after growth in MRS broth supplemented with 65 mM 1,2-PD and 15 mM glucose at 37°C for 18 h. (b) Extracted metabolosomes from cells grown as for panel a. (c) Enlarged view of metabolosomes shown in panel d. Arrowheads indicate a single-layer shell. (d) *L. reuteri* grown on MRS broth supplemented with 65 mM 1,2-PD at 37°C for 18 h. Arrows indicate metabolosomes.

**FIG. 3.** SDS-PAGE separation of *L. reuteri* total cell protein fractions and MALDI-TOF-identified proteins. Lane 1, protein molecular weight marker. Lane 2, diol dehydratase-positive fraction 1. Lane 3, diol dehydratase-positive fraction 2 (immediately below fraction 1 in sucrose density gradient). *L. reuteri* was grown in MRS broth supplemented with 15 mM glucose and 50 mM 1,2-PD at 37°C for 36 h.
DNA sequence analysis. Genes resembling *S. enterica* serovar Typhimurium linked *cob-pdu* operons were found in *L. reuteri* JCM 1112/DSM 20016 (Refseq NZ AAOV00000000) (Fig. 5), *L. brevis* ATCC 367 (Refseq CP000416) (24), and *L. hilgardii* (locus AY061969). *L. reuteri* 100-23 did not contain genes resembling the *S. enterica* serovar Typhimurium *cob-pdu* operons. A CRE sequence was detected in the *pocR-pduA* intergenic interval of *L. reuteri* DSM 20016 (TTGTAAGCCTAAGGTTT) and *L. collinoides* (TTGTAAGCCTAAGGTTTACT). MEME detected the consensus motif GAAGGCCTT when applied to the data set of the *pduA-pocR* intergenic sequences of *L. reuteri*, *L. brevis*, *L. collinoides*, and *L. hilgardii*. This corresponds to part of the CRE consensus sequence. The transcription start site of the *L. reuteri* *pduA* gene on induction by 1,2-PD was immediately upstream of the identified CRE sequence (Fig. 5, 6).

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

### DISCUSSION

We present the first demonstration that the antimicrobial agent-producing organism *L. reuteri* has the capacity to synthesize a bacterial microcompartment (carboxysome or metabolosome). The organism produced a cobalamin-dependent diol dehydratase induced by 1,2-PD, as in gram-negative bacteria containing the *pdu* operon. Linked cobalamin synthesis and propanediol utilization operons were present in the *L. reuteri* DSM 20016 genome sequence, and the entire *pdu* (propanediol utilization) operon was amplified from a laboratory strain of *L. reuteri* DSM 20016 by PCR, confirming its presence in the propanediol-metabolizing organism. Dismutation of 1,2-PD has been reported from another *Lactobacillus* sp., *Lactobacillus diolivorans*, *Lactobacillus buchneri*-like organism from maize silage (19). However, no assay of cobalamin production was reported, and metabolosomes were not seen on electron microscopy of *L. diolivorans* growing on medium incorporating 1,2-PD.

The conversion of 1,2-PD to propanol and propionate with the transient presence of propionaldehyde we have observed (Fig. 4b) suggests a pathway as described for 1,2-PD utilization in *Salmonella* (8, 22, 36) (Fig. 7). Genes specifying all the enzymes required (Fig. 7) were present in the *L. reuteri* F275 (DSM 20016) *pdu* operon (Fig. 5).

However, in *Enterobacteriaceae* like salmonellae capable of 1,2-PD utilization via a metabolosome-associated diol dehydratase, there are significant further onward metabolic connections for the dismutation products which are not present in lactobacilli. In *Salmonella*, the propionate product of 1,2-PD utilization can be coupled via the methylcitrate cycle to aerobic respiration (15, 28) or tetraionate reduction (30), allowing growth on 1,2-PD as a sole carbon and energy source. In the absence of oxygen or tetraionate, *Salmonella* spp. can grow only on defined no-carbon media containing added 1,2-PD to which yeast extract has also been added (30). It is proposed that this represents fermentative growth using a carbon source in the yeast extract with energy from propanediol dismutation (30). The pathways by which *Salmonella* spp. utilize propionate have not been observed in *Lactobacillus* spp., and no evidence for them is apparent from the *L. reuteri* genome sequence. We observed a steady increase in propionate levels in culture supernatant from *L. reuteri* 20016 grown in MRS medium with 1,2-PD over 8 days of continuous culture, suggesting that propionate is excreted and cannot be utilized by the organism. Lower growth rates were seen in MRS-MOD medium when 1,2-PD alone was added to the basal medium than when glucose was also added, but there was an advantage compared with the basal MRS-MOD medium (Fig. 4). It is likely that, as in nonrespiring *Salmonella* spp., *L. reuteri* growth on MRS-MOD medium with 1,2-PD is a result of fermentation of other

### TABLE 2. Peptide mass fingerprinting of metabolosome components

<table>
<thead>
<tr>
<th>Predicted mol wt</th>
<th>Identity assigned in <em>L. reuteri</em> F275 genome (locus tag)</th>
<th>NCBI accession no.</th>
<th>Mascot search result*</th>
<th>Coverage %</th>
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<tr>
<td>62,566</td>
<td>Glycerol dehydratase, large subunit PduC (Lreu 1747)</td>
<td>gi 148544953</td>
<td>19</td>
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<td>gi 148544946</td>
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<td>67</td>
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<tr>
<td>17,007</td>
<td>Protein of unknown function DUF336/PduOAbis (Lreu 1736)</td>
<td>gi 148544942</td>
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</tbody>
</table>

* Mascot search was used to compare the MALDI-TOF MS data obtained for sample proteins to predicted spectra for proteins present in the National Center for Biotechnology Information (NCBI) database. The Mascov score is the probability that the observed match is a random event. Protein scores greater than 80 are significant (P < 0.05).

### TABLE 3. 3-HPA (reuterin) production from glycerol and/or propanediol in 1 h by *L. reuteri* strains induced with glycerol or with glycerol and 1,2-PD

<table>
<thead>
<tr>
<th>MRS-MOD supplement</th>
<th>3-HPA concn (mM)a</th>
<th>3-HPA concn (mM)b</th>
<th>3-HPA concn (mM)b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. reuteri</em> F275</td>
<td>DSM 20016</td>
<td>DSM 100-23</td>
</tr>
<tr>
<td></td>
<td>L. reuteri 20016</td>
<td>L. reuteri 100-23</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.08</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Glycerol + 1,2-PD</td>
<td>0.08</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>1,2-PD</td>
<td>0.00</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Glycerol + 1,2-PD</td>
<td>0.46</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>1,2-PD</td>
<td>0.08</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

* Overnight induction conditions: 1,2-PD in all cases at 50 mM; glycerol induction, 20 mM; 200 mM glycerol for 3-HPA production assay conditions.

b Measured by dehydration to acrolein prop-2-enal.
carbon sources such as yeast extract in the complex MRS medium combined with energy from 1,2-PD dismutation (Fig. 7). The control organism L. reuteri 100-23 (in whose genome sequence no pdu operon is apparent) gained no growth advantage from the addition of 1,2-PD to the basal medium and was able to metabolize only a small amount of 1,2-PD over a period of 8 days (Fig. 4).

The enzyme specified by the pdu operon pduCDE genes, diol dehydratase, is responsible for conversion of 1,2-PD to the intermediate propionaldehyde. Interestingly, the enzyme responsible for glycerol conversion to 3-HPA in L. reuteri (Fig. 1) was previously described as a glycerol dehydratase (47) but is capable of acting as a propanediol dehydratase (47). The presence of two isofunctional related enzymes (glycerol and propanediol dehydratase) in L. reuteri, as in K. pneumoniae, was inferred from the existence of two peaks of propanediol dehydratase activity on cell extracts separated by DEAE-cellulose chromatography (47). This left the possibility that reuterin production could be dependent on either one of two isofunctional enzymes. The L. reuteri F275 (DSM 20016) genome sequence has recently been circularized (http://genome.jgi-psf.org/finished_microbes/lacre/lacre.info.html), and BLAST searching does not reveal a distinct glycerol dehydratase in addition to the diol dehydratase linked with cobalamin synthesis. That is, the only can-

FIG. 4. Growth characteristics of L. reuteri strains and anaerobic propanediol metabolism. (a) Growth curves of L. reuteri 20016 and L. reuteri 100-23 in MRS-MOD at the indicated times postinoculation. O.D.: optical density at 600 nm. ▲—▲, L. reuteri 20016 with 50 mM glucose. △—△, L. reuteri 20016 with 50 mM 1,2-PD. ★—★, L. reuteri 20016, unsupplemented. ○—○, L. reuteri 100-23 with 50 mM 1,2-PD. ○—○, L. reuteri 100-23 with 50 mM glucose. □—□, L. reuteri 100-23 with 50 mM 1,2-PD. (b) Propanediol metabolism by L. reuteri 20016 or L. reuteri 100-23 in MRS-MOD with 1,2-PD at the indicated times postinoculation. Metabolite concentrations are shown. ■—■, propanediol concentration in bacterium-free control. ○, L. reuteri 100-23, propanediol concentration. △—△, L. reuteri 20016, propanediol concentration. ▲, L. reuteri 20016, propionate concentration. ★, L. reuteri 20016, propionaldehyde concentration.

FIG. 5. pdu operon of L. reuteri. Predicted open reading frame gene assignment by comparison with S. enterica serovar Typhimurium (nomenclature of labeled cobalamin synthesis genes follows Salmonella convention). Gene cluster generated with 3BASE sequence file viewer (http://3base.bham.ac.uk/cgi-bin/fileprepare.cgi). The consensus CRE sequence is boxed, predicted −35 and −10 promoter sequences and the ribosomal binding site are underlined, and the start codon of pduA is in bold. The transcriptional start site when induced by propanediol is in larger type. The extent of PCR products and predicted restriction sites are shown below the operon.
didate enzyme identifiable from the genome sequence for produc-
tion of 3-HPA from glycerol forming the antimicrobial
reuterin (56) is the metabolosome-associated propanediol-in-
duced diol dehydratase we describe. Supporting this, we found no
phenotypic evidence of a distinct glycerol-induced dehydratase in
L. reuteri 20016 (Table 1), and maximal reuterin production by
L. reuteri 20016 was associated with preincubation with 1,2-PD in
addition to glycerol (Table 3). Very small amounts of reuterin
were produced in the absence of 1,2-PD under preincubation or
assay conditions (Table 3).

L. reuteri 100-23, lacking the metabo-
losome-associated diol dehydratase in its unpublished genome
sequence, was unable to synthesize more than trace amounts of
reuterin (at most, less than 6% of that detected from
L. reuteri 20016) (Table 3) and had very low levels of diol dehydratase
activity, irrespective of substrate induction (Table 1).

While appearances of individual metabolosomes were con-
sistent with electron microscopy reports on Salmonella (8, 14,
41), fewer metabolosomes were observed in each bacterial cell,
and metabolosomes were agglomerated (Fig. 2a, c, and d).
Similar electron microscopy appearances have been reported
from L. collinoides (38), which also expresses a metabolosome-as-
associated diol dehydratase (39) but does not synthesize co-
balamin. Biochemical data supported these qualitative elec-
tron microscopy appearances, showing a reduced specific
enzyme activity compared with gram-negative organisms: max-
imal diol dehydratase activity per mg of whole cell extract was
comparable with that reported for L. collinoides (39) and ap-
proximately a quarter of that reported for Salmonella (14).

Although the pdu operon is substantially similar in gene
number and order in Salmonella and L. reuteri, DNA sequence
analysis upstream of the pdu operon suggests that it may be
regulated differently (Fig. 4). The linked cob and pdu metabo-
losome operons in a gram-negative background are regulated
by Crp and Arc (1). In Lactobacillus spp., as for other gram-
positive organisms (35), catabolite repression generally occurs
via HPr [HPr(Ser-P)], the small phosphocarrier protein of the
phosphoenolpyruvate-sugar phosphotransferase system, and
CcpA protein (6, 17), operating via short CREs in the DNA
sequence (4, 17, 25). Although 1,2-PD utilization operons have
been described for other Lactobacillus species, CREs have not
previously been noted in connection with them. We identi-
fied a CRE consensus sequence in the L. reuteri pdu operon up-
stream of pduA, the first gene in the pdu operon. We found
complete or partial CRE sequences upstream of pduA in all
other available DNA sequences from Lactobacillus spp. con-
taining this operon. In L. reuteri, the center of the CRE is 17 bp
downstream of the transcription start site of the initial gene in
the pdu operon when induced by 1,2-PD and +22 bp relative to
the end of the putative −10 sequence (Fig. 5). In Lactococcus
lactis, a CRE in this orientation is associated with strong CcpA-
dependent repression (59).

The requirement for a complex 22-gene 1,2-PD utilization
operon for this apparently simple process has been attributed
to the need to contain the intermediate compound propional-
dehyde within a protein compartment or metabolosome, either
to reduce toxicity (36) or to prevent its loss as a gas by the cell
(29). As reported for metabolosome-containing S. enterica me-
tabolizing 1,2-PD (36), we detected only small amounts of
propionaldehyde in culture supernatants of 1,2-PD-metaboliz-
ing L. reuteri (Fig. 3b), suggesting retention within the metabo-
losome. It has been suggested that in the metabolosome asso-
ciated with the ethanolamine utilization operon in S. enterica,
the mechanism of aldehyde retention is based on reduced loss
of the aldehyde intermediate (in this case acetaldehyde) by evaporation, possibly by creating a low pH within the compartment, rendering aldehydes more likely to convert to a less volatile acetal (29). However, with regard to the S. enterica 1.2-PD utilization metabolosome, assays of pdu4 deletion mutants not producing the metabolosome shell but retaining metabolic activity showed that increased propionaldehyde evaporation was not a major factor affecting 1.2-PD metabolism (36). If, as we suggest, the metabolosome-associated diol dehydratase is also responsible for reuterin (3-HPA) production from glycerol, then the fact that this aldehyde is excreted by the organism suggests that either 3-HPA is not produced within the aldehyde-retaining metabolosome (i.e., a significant amount of diol dehydratase is outside the metabolosome in the cytoplasm, unlike the situation in Salmonella [8]) or the NAD-dependent oxidoreductase which removes 3-HPA in L. reuteri by conversion to 1,3-propanediol (Fig. 1) might not be localized in the metabolosome in the same way that PdUP coenzyme A-acylating propionaldehyde dehydrogenase is present within the 1,2-PD-metabolizing metabolosome (22) (Fig. 7). That is, effective aldehyde retention by the metabolosome requires the presence of specific aldehyde-metabolizing enzymes within the metabolosome.

Carboxymes in cyanobacteria affect internal cytoplasmic pH (7) and concentrate protons. There is recent evidence for regulation of the pdu operon by external pH in L. reuteri. During revision of the manuscript it was reported that gene transcription assays using a DNA microarray based on partial genome sequence data from L. reuteri ATCC 55730 showed that 11 genes from the pdu operon were downregulated by dilution and incubation at pH 5.1 versus pH 2.7 (57). Lactobacilli, including Lactobacillus reuteri, are heterotrophic fermentative organisms that obtain energy from the pHi of the remaining cytoplasm, compromising efforts to study the constraints of operating in a fermentative background will dramatically transmitted between different bacteria. Further study of the constraints of operating in a fermentative background will shed new light on the electrochemical properties of the metabolosome.

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References
