The complete coenzyme B$_{12}$ biosynthesis gene cluster of *Lactobacillus reuteri* CRL1098

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The coenzyme B$_{12}$ production pathway in *Lactobacillus reuteri* has been deduced using a combination of genetic, biochemical and bioinformatics approaches. The coenzyme B$_{12}$ gene cluster of *Lb. reuteri* CRL1098 has the unique feature of clustering together the cbi, cob and hem genes. It consists of 29 ORFs encoding the complete enzymic machinery necessary for de novo biosynthesis. Transcriptional analysis showed it to be expressed as two tandem transcripts of approximately 22 and 4 kb, carrying cobD, cbiABCDETFGHJ, cobA/hemD, cbiKLMNQOP, sirA, hemACBL, and cobUSC, cobT, respectively. Both transcripts appear to be similarly regulated, and under the conditions assayed are induced in the late-exponential growth phase. Evidence for a regulatory mechanism of negative feedback inhibition by vitamin B$_{12}$ itself was observed. Comparative genomics analysis of the coding sequences showed them to be most similar to those coding for the anaerobic coenzyme B$_{12}$ pathways previously characterized in a few representatives of the genera *Listeria* and *Salmonella*. This contrasts with the trusted species phylogeny and suggests horizontal gene transfer of the B$_{12}$ biosynthesis genes. G+C content and codon adaptation index analysis is suggestive that the postulated transfer of these genes was not a recent event. Additional comparative genomics and transcriptional analysis of the sequences acquired during this study suggests a functional link between coenzyme B$_{12}$ biosynthesis and reuterin production, which might be implicated in *Lb. reuteri*'s success in colonizing the gastrointestinal tract. This information on gene organization, gene transcription and gene acquisition is relevant for the development of (fermented) foods and probiotics enriched in B$_{12}$.

INTRODUCTION

*Lactobacillus reuteri* is a Gram-positive, heterofermentative lactic acid bacterium, widespread throughout the gastrointestinal tract (GI tract) of humans and other animals (Walter et al., 2003). Although this bacterium is currently marketed as a probiotic, human intervention studies showing relevant benefits remain to be reported (Saxelin et al., 2005). Nonetheless, potential probiotic effects have been demonstrated; they include lowering blood cholesterol levels in mice (Taranto et al., 2000), and stimulating anti-inflammatory activity in human cell lines (Ma et al., 2004).

*Lb. reuteri* possesses the unique ability to produce and excrete reuterin (Talarico et al., 1988). This broad-spectrum antimicrobial compound is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) (Talarico & Dobrogosz, 1989). The synthesis of reuterin is mediated by glycerol dehydratase (EC 4.2.1.30), a vitamin B$_{12}$-dependent enzyme, which is involved in catalysing the conversion of glycerol to 3-HPA (Daniel et al., 1998).

We have reported the isolation of a compound from *Lb. reuteri* CRL1098 capable of fulfilling the auxotrophic B$_{12}$ requirements of three different indicator strains (Taranto et al., 2003). In the same study, *Lb. reuteri* genomic DNA was found to contain sequences homologous to genes involved in the anaerobic coenzyme B$_{12}$
biosynthesis pathway, including \textit{cysG}/\textit{hemD} from \textit{Selenomonas ruminantium} (Anderson et al., 2001), \textit{Listeria innocua} and \textit{Listeria monocytogenes} (Glaser et al., 2001), and \textit{cblK} and \textit{cblJ} from \textit{Salmonella typhimurium} (Raux et al., 1996).

Vitamin B\textsubscript{12} consists of a tetrapyrrolic-derived corrin ring with a cobalt ion chelated at the core. Along with chlorophyll, coenzyme F\textsubscript{430} and haem, amongst others, it constitutes one of the most structurally complex classes of cofactors. Various B\textsubscript{12} derivatives with different upper axial ligands act as essential cofactors in many important enzymatic reactions responsible for the catalysis of methyl transfers and carbon backbone rearrangements (Maggio-Hall & Escalante-Semerena, 1999). Coenzyme B\textsubscript{12} biosynthesis is limited to a few representatives of bacteria and archaea (Martens et al., 2002). It appears that B\textsubscript{12}-dependent enzymes are absent from plants and fungi, but widespread in prokaryotes, protists and animals (Croft et al., 2005; Rodionov et al., 2003).

In humans, vitamin B\textsubscript{12} deficiency leads to pernicious anaemia and neurological dysfunction, amongst other complications (Stabler, 1999). Three proteins are known to participate in the uptake and transport of vitamin B\textsubscript{12}, namely haptocorrin, intrinsic factor and transcobalamin II. Absorption of vitamin B\textsubscript{12} occurs by receptor-mediated endocytosis in the terminal ileum, where the specific receptor cubulin complexes with intrinsic factor bound to B\textsubscript{12} (Banerjee, 2006). As a consequence, B\textsubscript{12} produced by colonic bacteria is most likely inaccessible to the host. However, it has been suggested that B\textsubscript{12} produced by a micro-organism capable of colonizing proximal to the ileum, such as \textit{Lb. reuteri}, would potentially be host-accessible (Albert et al., 1980).

\textit{Lb. reuteri} was the first lactic acid bacterium reported to be able to produce B\textsubscript{12} (Taranto et al., 2003). Increasing our understanding of how this GRAS (generally regarded as safe) organism encodes, acquired and maintains a biosynthetic pathway of such complexity and magnitude is of great importance to the medical field and for the food and feed industries.

In this study, we extend the analysis of the presumed coenzyme B\textsubscript{12} biosynthesis gene cluster of \textit{Lb. reuteri} and describe the presence of a complete gene cluster encoding all the enzymic machinery necessary for the de novo synthesis of this important cofactor. Additional comparative genomics and transcriptional analysis of the new sequences acquired during this study suggests a functional link between coenzyme B\textsubscript{12} biosynthesis and reuterin production, which might be implicated in \textit{Lb. reuteri}'s success in colonizing the GI tract.

**METHODS**

**Strains, media and culture conditions.** \textit{Lb. reuteri} CRL1098, isolated from sourdough, was obtained from the CERELA stock culture collection. It was cultivated at 37 °C in MRS medium and in vitamin B\textsubscript{12} assay medium (Sigma) supplemented when mentioned with 1 mg L\textsuperscript{-1} of cyanocobalamin (Sigma-Aldrich). \textit{Escherichia coli} strain XL-1 Blue MRA (P2) was obtained from Stratagene and cultivated at 37 °C under aerobic conditions in TY medium. \textit{Salmonella enterica} serovar Typhimurium LT2 derivative strains TT25720 \textit{(metE2119::MudJ)} and TT25722 \textit{(metE2119::MudJ, cobs2621::Fr(sw)} were kindly provided by Professor John R. Roth (Section of Microbiology, University of California at Davis, USA) and cultivated at 37 °C in TY medium or minimal E medium (Maloy et al., 1996), supplemented with 100 nM cyanocobalamin when required.

**Nucleotide sequence analysis.** The sequence of the B\textsubscript{12} biosynthesis gene cluster of \textit{Lb. reuteri} was obtained by screening two genomic \textit{λ}-phage libraries, and finalized by both inverted PCR and genomic primer walking. Total genomic DNA was isolated from \textit{Lb. reuteri} according to standard molecular biology techniques (Sambrook & Russell, 2001).

A Southern blot analysis of a partial digestion of \textit{Lb. reuteri}'s chromosomal DNA with the restriction enzymes \textit{BglII} and \textit{BamHI}, using a \textit{cysG}/\textit{hemD} (Taranto et al., 2003) probe amplified from the same strain, showed that the signals obtained corresponded to DNA fragments larger than 15 kb for both restriction enzymes (data not shown). Based on this knowledge, two \textit{Lb. reuteri} genomic \textit{λ}-phage libraries were constructed by the separate ligation of \textit{BglII}- and \textit{BamHI}-digested \textit{Lb. reuteri} genomic DNA into Lambda-DASH \textit{II}, \textit{BamHI} vector and packaged with a Gigapack III Gold packaging extract (Stratagene) according to the manufacturer’s recommendations. For the amplification of the Lambda-DASH \textit{II/BamHI} libraries a lysogenic P2 strain, \textit{E. coli} XL-1 Blue MRA (P2), was used. Titre determination of bacteriophages, blotting of plaques on nylon membranes and \textit{λ}-DNA isolation were all performed according to the manufacturer’s recommendations. Probes purified through the JETPURE PCR Product Purification kit (GENOMED), were amplified, radioactively labelled with \textit{[α\textsuperscript{32P}]ATP} (GE Healthcare Europe), and hybridized on membranes according to standard procedures (Sambrook & Russell, 2001). Membranes were exposed to BioMax MS or BioMax MR X-ray film (Kodak) for at least 5 h at −80 °C before developing. Sequencing of two −15 kb non-overlapping inserts containing B\textsubscript{12}-related DNA was carried out at Greenomics (Wageningen, The Netherlands).

For gap-closure between the two inserts, we resorted to inverted PCR. By standard procedures (Sambrook & Russell, 2001), \textit{HindIII}-digested genomic DNA of \textit{Lb. reuteri} was ligated to pNZ8048 (Sybesma et al., 2003) digested with the same endonuclease. The ligation mixture was directly used as a template in a PCR using a primer designed on the vector and another based on the 5’ flanking region of the known sequence at the time. The resulting amplicon was isolated from an agarose gel and sequenced directly at Baseclear, The Netherlands.

Further sequencing efforts aimed at closing gaps and extending the flanking regions of the known sequence were done by genomic primer walking carried out at GATC Biotech, Germany.

The new sequence information obtained using the three different approaches described above was analysed and assembled resorting to in-house scripts, and online programs available from the Biology WorkBench of the San Diego Supercomputer Centre (http://workbench.sdsc.edu/). Standard RNA regulatory motif searches were performed in Rfam (Griffiths-Jones et al., 2003) and using Riboswitch finder (Bengert & Dandekar, 2004). Predicted ORFs were manually annotated based on homology searches using the \textsc{blast} algorithm (Altschul et al., 1997). All sequence information was deposited at GenBank under accession no. AY780645.

**Complementation studies.** A fragment containing \textit{cobs} was amplified from \textit{Lb. reuteri}'s genomic DNA using Herculase II DNA polymerase.
polymerase (Stratagene), and primers LRE28196_28215 and LRE29724_29704 (Tables 1 and 2). Additionally, a fragment containing the native cob operon promoter (O’Toole et al., 1993) was amplified from Salmonella enterica strain TT25720 using primers 5’-GACCACTTGGATTGAGTGGGAC-3’ and 5’-GATGATC-GATCATACGGGTCTCCGTAGT-3’ (Clad cleavage site underlined). 3’-A overhangs were added to both fragments by incubating the PCR reactions directly with 1 unit of Taq DNA polymerase for 5 min at 72 °C. The A-tailed fragments were then purified with the JETPURE PCR Product Purification kit (GENOMED) and digested with Clad. The modified fragments were again purified by the same method and simultaneously cloned in pGEM-T Easy Vector (Promega Benelux), resulting in pNZ7749. The Salmonella enterica strain TT25722 was transformed with this vector as previously described (Sambrook & Russell, 2001), and its phenotype was characterized in minimal E medium (Maloy et al., 1996).

Transcriptional analysis. The transcriptional organization of the vitamin B₁₂ gene cluster of Lb. reuteri was determined by Northern blots, reverse transcriptase PCR (RT-PCR) and quantitative RT-PCR (Q-RT-PCR). Cells were cultured in batch fermentations of MRS medium, vitamin B₁₂ assay medium (commercial rich broth lacking B₁₂) and vitamin B₁₂ assay medium supplemented with vitamin B₁₂ to a final concentration of 1 mg l⁻¹. RNA was isolated according to standard procedures (Sambrook & Russell, 2001) from samples collected at the mid-exponential, late-exponential and stationary phases. The integrity and concentration of the RNA were analysed with a 2100 Bioanalyser (Agilent Technologies). Northern blotting of RNA obtained from late-exponential-phase cells cultivated in MRS was performed as previously described (Kuipers et al., 1993; Roest et al., 2005). Probes were amplified from genomic DNA of Lb. reuteri by PCR using primer pairs designed to locate them throughout the cluster, notably on divC, chp and cobT. Subsequent hybridization with radiolabelled probes was carried out according to standard techniques (Sambrook & Russell, 2001). RT-PCR analysis of samples obtained from cells cultured in MRS was performed by systematically amplifying overlapping fragments throughout the full extent of the B₁₂ biosynthesis cluster and flanking regions. All RNA samples were diluted to the same concentration and an extra DNase I (Invitrogen) treatment was implemented to eliminate possible remaining chromosomal DNA contamination. First-strand cDNA synthesis was carried out using Superscript III reverse transcriptase from Invitrogen according to the manufacturer’s recommendations. Primers were manually designed and are listed in Table 1. To quantify the differential expression of the two operons within the B₁₂ biosynthesis gene cluster between late- and mid-exponential phases and in the presence or absence of B₁₂, we performed Q-RT-PCR. Amplification was carried out in 96-well plates in an ABI Prism 7700 (Applied Biosystems) using the fluorescent agent SYBR Green for detection. Reactions were set up using the SYBR Green Master Mix from the same manufacturer, following its recommendations. Specificity and product detection were checked after amplification by determining the temperature-dependent melting curves. Primers were designed with the Primer Express software package (Applied Biosystems) to have a Tm between 59 and 61 °C and an amplicon size of 100 ± 20 bp (Table 2). Comparisons were made between the different growth phases and the different culture media.

Phylogenetic analysis. Each individual B₁₂-related amino acid sequence reported in this study was entered as a string to search for distantly related homologues using the PSI-BLAST algorithm (Altschul et al., 1997). Sequence entries identified as homologues were retrieved from March 2007 from ERGO (http://ergo.integratedgenomics.com/ERGO/) (Overbeek et al., 2003), and separately aligned using the MUSCLE algorithm (Edgar, 2004). From the sequence alignment of the proteins encoded by the coenzyme B₁₂ biosynthesis cluster, a neighbour-joining tree was obtained using CLUSTAL W (Thompson et al., 1994), analysed in LOFT (van der Heijden et al., 2007), and visualized in MEGA3 (Kumar et al., 2004). An identical exercise was carried out for the predicted product of the rpsO gene that is located downstream of the vitamin B₁₂ gene cluster of Lb. reuteri, and for the 16S RNA gene. Finally, the topology of all trees was compared.

G+C content and codon adaptation index. G+C content and codon adaptation index (Sharp & Li, 1987) was calculated using the geecce, cusp and cai scripts, part of EMBoss: European Molecular Biology Open Software Suite (Rice et al., 2000). Comparisons were made between the coenzyme B₁₂ biosynthesis gene cluster of Lb. reuteri presented here and the draft genome sequence of Lb. reuteri JCM1112 obtained by the DOE Joint Genome Institute and deposited at GenBank under accession no. CP000705. A similar exercise was performed for Listeria innocua Clp11262 (Glaser et al., 2001) and Salmonella enterica Typhi Ty2 (Deng et al., 2003), for which we compared the G+C content and codon usage of their vitamin B₁₂ clusters to their published genomes.

RESULTS

Operon organization

A sequence of approximately 43.4 kb was assembled from the Lb. reuteri genome through the combined effort of the different molecular biology techniques, and was found to harbour a coenzyme B₁₂ gene cluster encoding the complete enzymic machinery necessary for its biosynthesis. An overview of the organization of this gene cluster (Fig. 1) reveals that all predicted genes are in the same orientation, with only a few intergenic regions. Similar to what has been reported for Salmonella typhimurium (Roth et al., 1993), we observed that approximately half of the genes (46%) are overlapping and predicted to be translationally coupled.

The previously published sequence encoding the fusion protein homologous to CysG/HemD (Taranto et al., 2003) is flanked by the large cluster of 17 cbi genes (Fig. 1). The cbi gene order is conserved amongst different B₁₂ producers, notably representatives of Listeria and Salmonella (see Fig. 1). Quite unexpectedly, the hem genes are located directly downstream of the cbi genes. To our knowledge this genomic organization has not been described previously. These genes are predicted to encode uroporphyrinogen III synthesis from 5-aminolaevulinate, a derivative of glutamyl-tRNA. A cluster of five cob genes is located further downstream. This cluster is predicted to be involved in the attachment of the amino-propanol arm and assembly of the nucleotide loop, which connects the lower cobalt ligand to the corrin ring. Upstream of the B₁₂ biosynthesis gene cluster are several genes predicted to be involved in the formation of polyhedral bodies, including pduU and pduV (Bobik et al., 1999).

Detailed comparison of the predicted coding sequences of Lb. reuteri CR1L098 and the draft genome sequence of Lb. reuteri JCM1112, recently released by the DOE Joint Genome Institute, demonstrates that they are mostly identical (Table 3). The few exceptions are due to minor changes in the N-terminus (CbiA and CbiB), or in the C-
Table 1. Oligonucleotide primers used in RT-PCR reactions

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*Primers were designed to the coding strand (+) or non-coding strand (−).
†Primer also used in Q-RT-PCR experiment.
‡Primer also used in complementation studies.
terminus (CbiD and CobD), or due to the neutral replacement of residues with the same chemical properties (CbiC and CobU).

**Complementation studies**

To experimentally support our functional annotation of the newly sequenced coenzyme B$_{12}$ biosynthesis gene cluster of *Lb. reuteri*, we performed complementation studies in *Salmonella enterica* mutants TT25720 (metE2119::MudJ) and TT25722 [metE2119::MudJ, cobS2621::Frt(sw)] (see Methods). When cultured in minimal medium lacking methionine, both strains are dependent on the B$_{12}$-dependent methionine synthase (MetH), since they lack MetE activity. However, due to the additional cobS mutation, strain TT25722 has auxotrophic requirements for B$_{12}$, while strain TT25720 can rely on its own native production of this cofactor.

We transformed the double mutant TT25722 with pNZ7749, harbouring a fragment containing cobS amplified from *Lb. reuteri* under control of the native cob operon promoter from *Salmonella enterica* (O’Toole et al., 1993). Growth experiments were then performed on minimal E plates using strains TT25720 and TT25722 as a positive and negative control, respectively. Complementation of the double mutant with cobS from *Lb. reuteri* reconstituted its ability to grow in minimal medium lacking methionine without the exogenous supplementation of vitamin B$_{12}$, and therefore relying solely on its own native coenzyme B$_{12}$ production (Fig. 2).

**Transcription analysis**

In order to determine the transcriptional organization of the B$_{12}$ biosynthesis gene cluster we performed Northern blot analysis (see Supplementary Fig. S1, available with the online version of this paper). As a consequence of the relative rarity of the transcripts encoding B$_{12}$ biosynthesis enzymes and their remarkably large size, we could predict that technical difficulties with the Northern blots would not allow conclusive determination of the exact size of the different operons within this gene cluster. Nonetheless, probes were designed to be complementary to sequences from the beginning and end of the predicted operons and their use in Northern hybridizations revealed the presence of two transcripts, one with a size over 20 kb and another of 4 kb (Fig. S1).

To further characterize the transcriptional organization of this gene cluster, a RT-PCR based strategy was implemented. It consisted of systematically amplifying overlapping RT-PCR fragments throughout the full extent of the cluster and flanking regions. To validate the specificity of the designed primer pairs, all reactions were tested in parallel using chromosomal DNA of *Lb. reuteri* as a positive control. The absence of any chromosomal DNA contamination was established by carrying out all reactions.
Using RT-negative samples as a template, for a negative control. The results from the RT-PCR experiments (Table 4, Fig. 3) confirmed that the B12 biosynthesis gene cluster is expressed in two separate, but tandem, operons of approximately 22 and 4 kb. The large transcript includes the genes cobD, cbiABCDETFGHJ, cobA/hemD, cbiKLMNQOP, sirA and hemACBL. The 4 kb transcript derives from the cobUSC, hemD and cobT genes.

The intensities of the RT-PCR amplicons were compared between samples collected from the same MRS culture at different time points (see Fig. 3 for illustration). This suggested that for cells cultured in MRS the expression of the B12 gene cluster is strengthened during late-exponential phase in comparison to mid-exponential phase. The rpsO gene, located immediately downstream from the B12 gene cluster, served as a control for the transcriptional analysis. RT-PCR samples of this gene collected from the same culture at different time points showed that it is expressed constitutively throughout the growth curve, in contrast to the neighbouring B12 genes.

To quantify the differential expression first evidenced by the RT-PCR experiments, Q-RT-PCR was carried out on different loci throughout the entire cluster, using a locus on the rpsO gene as a reference. The results are in accordance with the previous RT-PCR-based observation, and confirm that for cells cultivated in MRS the cluster is indeed strongly induced during late-exponential growth (Fig. 4a). The operon carrying the cbi and hem genes is

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**Fig. 1.** Schematic representation of the vitamin B12 gene cluster of *Lb. reuteri*, and comparison of gene order with *Listeria innocua* and *Salmonella enterica*. The arrows represent genes that are involved in the synthesis of uroporphyrinogen III if depicted in blue; involved in the synthesis of adenosylcobinamide if depicted in orange; involved in the synthesis of the lower ligand if depicted in green; involved in cobalt transport if depicted in red; not related to B12 biosynthesis if depicted in grey; and not studied here if depicted in white. Functional annotation: pduO, ATP:Co(I)rrinoid adenosyltransferase (EC 2.5.1.17); cobD, threonine-phosphate decarboxylase (EC 4.1.1.81); cbiA, cobyrinic acid a,c-diamide synthase (EC 6.3.1.1); cbiB, adenosylcobinamide-phosphate synthase (EC 6.3.1.10); cbiC, precorrin-8X methylmutase (EC 5.4.1.2); cbiD, precorrin-5B C1-methyltransferase (EC 2.1.1.12); cbiE, precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiE (EC 2.1.1.132); cbiT, precorrin-6Y C5,15-methyltransferase [decarboxylating] subunit CbiT (EC 2.1.1.132); cbiF, precorrin-4 C11-methyltransferase (EC 2.1.1.133); cbiG, precorrin-5A C20-acyltransferase (EC 2.3.1.1); cbiH, precorrin-3B C17-methyltransferase (EC 2.1.1.131); cbiJ, precorrin-6X reductase (EC 1.3.1.54); cysG/hemD, uroporphyrin-III C-methyltransferase (EC 2.1.1.107)/uroporphyrin-III synthase (EC 4.2.1.75); cbiK, sirohydrochlorin cobaltochelatase (EC 4.99.1.3); cbiL, precorrin-2 C20-methyltransferase (EC 2.1.1.130); cbiM, cobalt transport protein; cbiN, cobalt transport protein; cbiO, cobalt transport protein; cbiP, adenosylcobyric acid synthase (EC 6.3.5.10); sirA, precorrin-2 dehydrogenase (EC 1.3.1.76); hemA, glutamyl-tRNA reductase (EC 1.2.1.1); hemC, porphobilinogen deaminase (EC 2.5.1.61); hemB, δ-aminolaevulinic acid dehydratase (EC 4.2.1.24); hemL, glutamate-1-semialdehyde 2,1-amimomutase (EC 5.4.3.8); cobU, adenosylcobinamide kinase (EC 2.7.1.156)/adenosylcobinamide-phosphate guanylyltransferase (EC 2.7.7.62); cobS, adenosylcobinamide-GDP ribazoletransferase (EC 2.7.8.26); cobC, z-ribazole-5′-phosphate phosphatase (EC 3.1.3.73); hemD, uroporphyrinogen-III synthase (EC 4.2.1.75); cobT, nicotinate-nucleotide–dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21).
upregulated $4.56 \pm 0.92$-fold during late-exponential growth when compared to mid-exponential phase. Similarly, the smaller operon carrying the cob and hemD genes is upregulated by a factor of $5.03 \pm 0.32$ between the late- and mid-exponential phases. The same approach was performed on two loci upstream of the B12 biosynthesis gene cluster predicted to encode PduU and PduV. We observed an average upregulation of $5.91 \pm 3.25$ for these transcripts, similar to that observed for the B12 gene cluster (Fig. 4b).

In order to confirm that the observed upregulation of the B12 biosynthetic genes during the late-exponential phase is not caused by the exhaustion of the vitamin B12 present in MRS, further experimentation was carried out. We analysed by Q-RT-PCR samples obtained from cells grown in B12 assay medium, which is B12 free, and in B12 assay medium supplemented with cyanocobalamin (Fig. 4b). In the absence of B12 we observed that during the late-exponential phase the cbi and hem operon was upregulated $6.89 \pm 0.93$, slightly more than what was observed for MRS.

### Table 3. ORFs of the coenzyme B12 biosynthesis gene cluster of Lb. reuteri CRL1098: comparison on amino acid level to Lb. reuteri JCM1112, Listeria monocytogenes and Salmonella typhimurium

<table>
<thead>
<tr>
<th>Lb. reuteri CRL1098</th>
<th>Lb. reuteri JCM1112</th>
<th>Listeria monocytogenes</th>
<th>Salmonella typhimurium</th>
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<tbody>
<tr>
<td>Name</td>
<td>Length (aa)</td>
<td>Identity (%)</td>
<td>Length (aa)</td>
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<tr>
<td>cobT</td>
<td>356</td>
<td>100</td>
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</table>

Fig. 2. Phenotypic characterization of complemented Salmonella mutant. Minimal E agarose plate with (1) Salmonella enterica TT25720 (metE2119::MudJ); (2) Salmonella enterica TT25722 [metE2119::MudJ, cobS2621::Frt(sw)]; (3) Salmonella enterica TT25722 pNZ7749 (harbouring cobS from Lb. reuteri); (4) empty.
In the presence of an excess of exogenous B₁₂ there was a 3.47 ± 0.90-fold change between the late- and mid-exponential growth phases for this same operon. Even though this upregulation is diminished in comparison to that in the absence of exogenous B₁₂, it is still quite considerable. Similar results were observed for the cob operon, upregulated 6.35 ± 0.73 and 3.31 ± 0.56 in the absence and presence of exogenous B₁₂, respectively. The upregulation in MRS of the transcript levels of the pdu loci was also observed in the absence of B₁₂ from the medium. For these we observed a fold change of 3.76 ± 2.2 in the absence of exogenous B₁₂ and 1.98 ± 0.68 when there was an excess of B₁₂.

To characterize in greater detail the specific impact of B₁₂ supplementation for each growth phase, additional comparisons were made between the cultures lacking exogenous vitamin B₁₂ (average fold change of 0.96 ± 0.08 for the cbi and hem operon, and 0.92 ± 0.08 for the cob operon). During the late-exponential phase, even though in both conditions the abundance of B₁₂ biosynthesis transcripts is increased relative to the mid-exponential phase, in the absence of vitamin B₁₂ supplementation, that in the absence of exogenous B₁₂, it is still quite considerable. Similar results were observed for the cob operon, upregulated 6.35 ± 0.73 and 3.31 ± 0.56 in the absence and presence of exogenous B₁₂, respectively. The upregulation in MRS of the transcript levels of the pdu loci was also observed in the absence of B₁₂ from the medium. For these we observed a fold change of 3.76 ± 2.2 in the absence of exogenous B₁₂ and 1.98 ± 0.68 when there was an excess of B₁₂.

### Table 4. Summary of RT-PCR reactions

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Product*</th>
<th>Size (bp)</th>
<th>Location</th>
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</thead>
<tbody>
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<td>LRE5899_5921</td>
<td></td>
<td>1467</td>
<td>IS, cobD</td>
</tr>
<tr>
<td>LRE6317_6340</td>
<td></td>
<td>1049</td>
<td>cobD</td>
</tr>
<tr>
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<td>1519</td>
<td>cobD, chiAB</td>
</tr>
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<td>LRE8843_8862</td>
<td></td>
<td>1594</td>
<td>chiBCD</td>
</tr>
<tr>
<td>LRE10416_10437</td>
<td></td>
<td>1531</td>
<td>chiDE</td>
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<td>LRE11947_11947</td>
<td></td>
<td>1503</td>
<td>chiETF</td>
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<td>1509</td>
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<td>1511</td>
<td>chiHI, cysG/hemD</td>
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<td>1495</td>
<td>cysG/hemD, chiK</td>
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<td>chiMNQO</td>
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</table>

*Symbols refer to relative abundance of products on agarose gel. Increasing number of ‘+’ corresponds to increasing intensity of band (see Fig. 3 for illustration); ‘−’ corresponds to absence of expected band, indicating that the two genes are not part of the same transcript.

**Fig. 3.** RT-PCR amplicons from different loci collected from MRS cultures at different time points. Lanes A, RT-negative samples (negative control); lanes B, sample collected at mid-exponential phase; lanes C, sample collected at late-exponential phase; lanes D, Lb. reuteri chromosomal DNA (positive control).
their induction is stronger. For the late-exponential phase, when we compared the levels for cells cultured in the absence of exogenous B12 in relation to those cultivated in its presence, we determined an average fold-change of 1.90 ± 0.22 for the cbi and hem operon, and 1.82 ± 0.08 for the cob operon.

**In silico analysis and comparative genomics**

We determined the phylogeny of each predicted individual amino acid sequence encoded by the B12 gene cluster. We then compared them amongst each other, and with the deduced protein sequence of a control gene, rpsO, for which we performed the same exercise. The RpsO protein tree resembled the canonical phylogenetic topology deduced from 16S rRNA sequences (see Supplementary Fig. S2, available with the online version of this paper). In contrast, the predicted B12 proteins of *Lb. reuteri* were found to repeatedly cluster together with those of the genus *Listeria*, and closely neighboured by those of the genus *Salmonella* and other closely related γ-Proteobacteria (see Fig. 5 for illustration). This is suggestive of a common origin for the coenzyme B12 production pathway in these organisms. Variations from the mentioned tree topology were observed for sirA, hemACBL and cobT, and are addressed in the Discussion.

Both the G+C content and codon adaptation index (Sharp & Li, 1987) of the B12 cluster were compared with the draft genome sequence of *Lb. reuteri* JCM1112. The average G+C content of the coenzyme B12 biosynthesis gene cluster (36 mol%) does not differ significantly from the average of the draft genome sequence of *Lb. reuteri* available at the date of analysis (39 mol%). Concerning codon usage, again we did not observe any significant differences between the coenzyme B12 gene cluster of *Lb. reuteri* and other *Lb. reuteri* sequences. The average codon adaptation index for the genes of this cluster was calculated to be 0.69 ± 0.026, and we did not detect the usage of any rare codon. We also compared the G+C content and codon usage of the B12 biosynthesis clusters of *Listeria innocua* Clip11262 (Glaser et al., 2001), 39 mol%, and *Salmonella enterica* typhi Ty2 (Deng et al., 2003), 56 mol%, with their published genomes, 38 mol% and 52 mol% respectively.

**DISCUSSION**

The biosynthesis of coenzyme B12 from uroporphyrinogen III, the last shared metabolic precursor of the various tetrapyrrolic cofactors, requires about 25 enzymes, and has two different routes described: (i) the aerobic pathway studied in *Pseudomonas denitrificans* (Battersby, 1994); and (ii) the anaerobic pathway partially resolved in *Salmonella enterica*, *Bacillus megaterium* and *Propionibacterium shermanii* (Roessner & Scott, 2006). This biosynthetic pathway is commonly divided into three parts: (i) the synthesis of uroporphyrinogen III from either glutamyl-tRNA or...
Fig. 5. Bootstrapped neighbour-joining phylogenetic tree of the CbiC protein.
glycine and succinyl-CoA; (ii) the corrin ring synthesis, which differs between the anaerobic pathway, starting with the insertion of cobalt into precorrin-2, and the aerobic pathway, where the cobalt chelation reaction occurs only after corrin ring synthesis; and (iii) the corrin ring adenylation, attachment of the amino-propanol arm and assembly of the nucleotide loop bridging the lower ligand to the cobalt at the core of the corrin ring.

In Lb. reuteri we have found all the genes necessary to encode the complete anaerobic biosynthesis pathway of coenzyme B₁₂. Remarkably, and unlike the situation in other B₁₂-producing prokaryotes studied, genes for all three parts of the B₁₂ biosynthetic pathway are clustered together in one continuous stretch of the chromosome. This presents a great advantage if considering metabolic engineering strategies aiming at transferring B₁₂ production capability, as has been done before for other complex B vitamins (Sybesma et al., 2004; Wegkamp et al., 2004).

Based on the homology paradigm, our functional annotation of the newly sequenced coenzyme B₁₂ biosynthesis gene cluster of Lb. reuteri was experimentally verified for cobS by the complementation of Salmonella mutant TT25722 (see Methods), lacking MetE and CobS activity. If cultured in minimal medium lacking methionine, this strain relies on the B₁₂-dependent methionine synthase (MetH), and has auxotrophic requirements for this cofactor. When we transformed TT25722 with pNZ7749, harbouring a fragment containing cobS amplified from Lb. reuteri, we reconstituted its ability to grow in minimal medium, depending on its own native coenzyme B₁₂ production, and indirectly showed the functionality of cobS from Lb. reuteri (Fig. 2). Another example of functional evidence can be found in the recent report of the crystal structure of the PduO-type ATP : Corrinoid adenosyltransferase (St Maurice et al., 2007) also sequenced within the course of this study.

Northern blotting and RT-PCR have shown that both the cbb genes, responsible for corrin ring synthesis, and the hem genes, responsible for the synthesis of uroporphyrinogen III, are transcribed together as part of a nearly 22 kb multicistronic operon. Although remarkably large, similar-sized transcripts have been detected in other lactic acid bacteria (van Krakenburg et al., 2000). The cob genes are clustered in the same orientation, but expressed in a different operon of approximately 4 kb, situated just downstream of the previously mentioned cbb and hem transcript (Fig. 1).

The results from the Q-RT-PCR experiment corroborated the hypothesis emergent from the RT-PCR studies, that the B₁₂ biosynthesis gene cluster is strongly induced during the late-exponential growth phase (Fig. 4a). Both operons are approximately five-fold upregulated in late-exponential when compared to mid-exponential growth, as determined by Q-RT-PCR for cells cultured in MRS broth. To ensure that the observed induction of the B₁₂ biosynthesis genes in the late-exponential phase is not due to the depletion of B₁₂ pools in MRS, we carried out additional experiments in B₁₂-free medium. We compared the induction of these genes between late- and mid-exponential phase, for cultures in the absence or presence of excess exogenous B₁₂. Although there was some variation in the levels of induction, it was clear that in all conditions assayed the B₁₂ biosynthesis transcripts are more abundant in the late-exponential than in the mid-exponential phase (Fig. 4b).

The lower induction of the B₁₂ biosynthesis genes during the late-exponential phase in the medium supplemented with B₁₂ (Fig. 4c) suggests the presence of a regulatory feedback mechanism that inhibits the biosynthesis of this costly co-factor when it is available from the environment. Vitamin B₁₂ metabolism has been shown to be often regulated by a conserved RNA structural element, known as riboswitch (Vitreschak et al., 2003). We searched the coenzyme B₁₂ biosynthesis gene cluster of Lb. reuteri for such conserved motifs using Rfam (Griffiths-Jones et al., 2003) and Riboswitch finder (Bengert & Dandekar, 2004), but none could be found. The presence of a transposase immediately upstream of the first gene of the B₁₂ cluster might have disturbed the riboswitch. The regulatory gene pocR (Bobik et al, 1992), which is often between the B₁₂ biosynthesis and pdu clusters, is not in this location in the chromosome of Lb. reuteri. In fact, this common regulator can be found at the far end of the adjacent pdu operon in the recently released genome of Lb. reuteri JCM11112. Its presence is in agreement with the experimental evidence gathered during this study suggesting co-regulation between the B₁₂ cluster and the pdu genes located immediately upstream. PocR has been shown to be an activator of the coenzyme B₁₂ biosynthesis cluster (Bobik et al., 1992), and is likely to be involved in the observed negative feedback phenomena. Furthermore, PocR itself has been shown to be activated under carbon and redox control (Ailion et al., 1993), which explains why we observed in all conditions assayed an induction of the B₁₂ biosynthesis cluster during the late-exponential in comparison to the mid-exponential phase.

The topology of the phylogenetic tree obtained for the predicted product of the rpsO gene (data not shown) is similar to the canonical phylogenetic trees deduced from 16S rRNA sequences (see Fig. S2). In contrast, the phylogenetic comparison of all predicted amino acid sequences related to B₁₂ biosynthesis showed that Lb. reuteri systematically clusters together with members of the genus Listeria, and closely neighbours the genus Salmonella and closely related Enterobacteriaceae. An illustration of a B₁₂ biosynthesis protein phylogenetic tree is here depicted for CbiC, which was found to follow this topological pattern (Fig. 5). Exceptions to this topology include the products of sirA and hemABCL, for which Lb. reuteri clusters with Listeria and related genera of Gram-positive bacteria, while the Enterobacteriaceae now cluster with other γ-Proteobacteria, probably because their hem genes are properly adapted to aerobic conditions as well. In
addition the CobT protein is not encoded by the Listeria genomes, which may have suffered gene loss, while Lb. reuteri still clusters with Salmonella and closely related genera.

Lb. reuteri was the first lactic acid bacterium reported to produce coenzyme B₁₂, and the recently released genome sequences of a dozen lactic acid bacteria show no traces of genes related to B₁₂ production. This observation, combined with the great differences in topology of the B₁₂-related trees and the canonical phylogenetic tree, suggests the acquisition of this capability by horizontal gene transfer. This promiscuity related to B₁₂ metabolism between some genera of the Firmicutes and γ-Proteobacteria has been noted before when the phylogeny of the B₁₂ regulatory motifs was being investigated (Vitreschak et al., 2003).

The G+C content of Lb. reuteri’s B₁₂ biosynthesis gene cluster does not clearly differ from the rest of its available genomic sequence, and the average codon adaptation index of this cluster is elevated, indicating that it is well suited to Lb. reuteri’s translational machinery. The same holds true for the B₁₂ gene homologues of Listeria and Salmonella, indicating that the postulated horizontal gene transfer is not a recent event.

Associated with its survival strategy, Lb. reuteri is capable of producing and excreting reuterin, a broad-spectrum antimicrobial (Talarico et al., 1988; Talarico & Dobrogosz, 1989). The production of this key component for its competitiveness is mediated by a B₁₂-dependent enzyme, glycerol dehydratase, responsible for catalysing the conversion of glycerol to 3-HPA, an intermediate of 1,3-propanediol in the glycerol catabolism pathway. The hypothesis that the acquisition of reuterin production and production of coenzyme B₁₂ was a single event is supported by the following observations: (i) the genes involved in reuterin production are located just upstream of the B₁₂ biosynthesis gene cluster; (ii) both sets of genes show similar phylogeny; and (iii) both sets of genes have similar expression patterns and seem to be part of the same regulon. This evolutionary event has presumably resulted in the speciation of Lb. reuteri from the other Lactobacillus species, and might have been important in its evolution to colonize the GI tract.

Lb. reuteri possesses the GRAS status and is an industrially relevant micro-organism. From a biotechnological point of view, the findings reported in this study can be applied for natural enrichment of (fermented) foods with B₁₂. Furthermore, they shed light on Lb. reuteri as a good candidate to investigate the possibility of in situ delivery of B₁₂ in the GI tract.

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REFERENCES


B12 biosynthesis in Lactobacillus reuteri


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