ORIGINAL RESEARCH PAPER

Cloning and heterologous expression of *Lactobacillus reuteri* uroporphyrinogen III synthase/methyltransferase gene (cobA/hemD): preliminary characterization

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Abstract

Purpose of work   To clone, express and characterize uroporphyrinogen III synthase/methyltransferase gene (*cobA/hemD*) from *Lactobacillus reuteri*.
Some strains of *Lb. reuteri* produce cobalamin (vitamin B_{12}). Cobalamin biosynthesis relies on the sequential action of more than 25 enzymes in a complex metabolic pathway. We have cloned, expressed and characterized the gene in *Lb. reuteri* that codes for the S-adenosy L-methionine uroprophyrinogen III methyltransferase/ synthase (CobA/HemD), a key bifunctional enzyme in the biosynthesis of cobalamin and other tetrapyrrols.

Keywords   Cobalamin · *Lactobacillus reuteri* · Uroprophyrinogen III methyltransferase · Vitamin B_{12}

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Introduction

Lactobacillus reuteri is a member of the gastrointestinal microbiota of several animals, including humans (Casas et al. 1998). Some strains of this genus possess probiotic properties (Taranto et al. 1998, 2000) and produce and excrete a broad-spectrum antimicrobial agent (reuterin) during anaerobic sugar-glycerol co-fermentation (Talarico and Dobrogosz 1989). The pathway for conversion of glycerol to 3-hydroxypropionaldehyde (reuterin precursor) is mediated through a cobalamin-dependent glycerol dehydratase (Daniel et al. 1998). Previously, we reported for the first time that *Lb. reuteri* CRL1098 is able to produce cobalamin de novo (Taranto et al. 2003; Santos et al. 2007). This vitamin is required for the anaerobic utilization of glycerol through the reaction of the glycerol dehydratase (Talarico et al. 1990; Daniel et al. 1998).

Using the glycerol dehydratase pathway *Lb. reuteri* can also use 1,2 propanediol as an energy source. As with *Salmonella*, glycerol dehydratase and other enzymes are protected in polyhedrical bodies called microcompartments or metabolosomes (Sriramulu et al. 2008).

Genetical and biochemical work have revealed the biosynthetic pathway for cobalamin (vitamin B_{12} (Warren et al. 2002). There are two different pathways: the aerobic and anaerobic routes. The aerobic pathway has been elucidated in *Pseudomonas denitrificans*, a strain used in the industrial production of vitamin B_{12} whereas the anaerobic path has been

more difficult to elucidate, and there are still some steps to be clarified. Anaerobic cobalamin synthesis has been studied mainly in *Salmonella enterica* and *Propionibacterium* sp. (Roessner et al. 2002; Frank et al. 2005).

In a recent collaborative work, we described the complete cobalamin biosynthetic cluster from *Lb. reuteri* CRL1098 (Santos et al. 2008). The availability of the genome sequences of two *Lb. reuteri* strains helped, applying physiological and *in silico* analysis, to reveal that this microorganism synthesizes B_{12} through the anaerobic pathway (Taranto et al. 2003; Santos et al. 2007). Curiously, the B_{12} biosynthesis cluster in *Lb. reuteri* DSM 20016 is physically associated with glycerol-metabolizing genes, strongly suggesting the presence of a glycerol-cobalamin genomic island in this microorganism (Morita et al. 2008). This observation can be extended to other *Lb. reuteri* strains with B_{12} biosynthesis genes whose genome sequences were recently released (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

Even though the amount of B_{12} produced by *Lb. reuteri* CRL 1098 (aprox. 10–50 μg/l) is significantly lower than those produced by the industrially B_{12}-producer bacteria *Propionibacterium shermanii* or *P. denitrificans* (B_{12} production > 100 mg/l) (Martens et al. 2002) the CRL 1098 strain is a proven probiotic food-grade microorganism whose ability to produce cobalamin confers an interesting additional feature on it. Supplementing a B_{12}-free diet with *Lb. reuteri* CRL 1098 was effective in preventing the pathologies caused by the nutritional deficiency of vitamin B_{12} both in pregnant mice and the weaned young (Molina et al. 2009). Although these results are encouraging, our investigations are directed to maximizing the synthesis of the vitamin, particularly in its active form in mammals. Therefore, it is important to begin the characterization of some key enzymes in the biosynthesis of this vitamin by *Lb. reuteri* CRL1098 in order to pave the way for future studies of metabolic engineering to increase the vitamin production and the amount of the active coenzyme. Recently, the *pduO* gene of *Lb. reuteri* CRL 1098 was over-expressed and the coding protein, PduO, was crystallized and characterized (St Maurice et al. 2007). This enzyme is involved in one of the last steps of B_{12} biosynthesis: the corrinoid

adenosylation, to give rise to the active coenzyme adenosyl-cobalamin (Escalante-Semerena 2007). In the present work, we report the cloning and heterologous expression of the uroporphyrinogen III-methyltransferase-synthase gene (*cobA/hemD*), which codifies for a key enzyme in the early steps of cobalamin biosynthesis.

Materials and methods

Bacterial strains, plasmids, and media

Lactobacillus reuteri CRL 1098, isolated from sour dough, belongs to CERELA culture collection. *Lactococcus (Lc.) lactis* NZ 9000, a plasmid-free strain with a high transformation efficiency, was used for electrotransformation and expression of the *cobA/hemD* gene from *Lb. reuteri* CRL 1098. *Escherichia (E.) coli* DH5α (Invitrogen) was used for routine DNA transformation and plasmid isolation. *Escherichia coli* BL21(DE3) was utilized for *cobA/hemD* overexpression, and *E. coli* SASZ31 [*hemD*] (Chartrand et al. 1979) was employed for complementation studies. *Lactobacillus reuteri* was grown in MRS broth or in B_{12}-free assay medium (Merck, Argentina). *Lactococcus lactis* was grown in M17 broth (Biokar Diagnostics, France) and incubated at 30°C without agitation. *E. coli* strains were routinely grown in Luria-Bertani broth (Sambrook et al. 1989) at 37°C with aeration or on LB supplemented with 1.5% agar. *E. coli* SASZ31 was grown in TBAB (Tryptose blood agar base, Difco) supplemented with hemin (2.5 mg/ml) and cysteine (20 mg/ml) (Johansson and Hederstedt 1999). Ampicillin (100 μg/ml) was added when appropriate. Vector pCR. 2.1 TOPO (Invitrogen) and plasmid pET28b (Novagen) were used for PCR fragment cloning, and subcloning, respectively.

DNA manipulations

General molecular biology techniques were carried out by standard procedures. *Lactobacillus reuteri* genomic DNA isolation has been performed as described by Pospiech and Neumann (1995). *Escherichia coli* plasmids isolated according to Birnboim (1983) or using a Wizard plasmid DNA purification system (Promega, Madison WI).

CobA/hemD gene cloning

Routine PCR amplification was performed with *Taq* DNA polymerase according to manufacturer's instructions in a thermocycler. For cloning PCR products, vector TOPO TA cloning kit (Invitrogen) was used routinely. Plasmid pTOPOCobA was constructed as follows: total genomic DNA from *Lb. reuteri* CRL 1098 was used to amplify the *cobA/HemD* gene with primers Cob-F (forward 5′-GGT CGCCAAGGTGGGGTTC-3′) and Cob8 (reverse 5′-CGTCTCTAATAAAGGCGCTG-3′), which were designed based on the sequence of the Cbl biosynthesis cluster (accession number AY780645.1). PCR amplification produced a 1.8 kb amplicon that was cloned in pCR 2.1 TOPO TA cloning vector and transformed in *E. coli* DH5α. Recombinant colonies were selected by blue/white screening. Several clones were analyzed for their plasmid content and one containing an insert with the correct size (plasmid pTOPOCobA) was selected for further experiments. The insert of this plasmid was sequenced in order to confirm its identity.

HemD complementation studies

To verify that CobA/HemD from *Lb. reuteri* CRL 1098 also has urogen III synthase activity, *E. coli* SASZ31 was transformed with plasmid pTOPO-CobA. This strain has a mutation in the *hemD* gene, and can grow in TBAB broth only when supplemented with hemin (2.5 mg/ml) and cysteine (20 mg/ml) (Johansson and Hederstedt 1999).

Microbial growth and cell lysates preparation

A single colony of the appropriate strain was inoculated into 22 ml Luria-Bertani (LB) medium. At the mid-growth phase, protein expression was induced by adding IPTG (0.5 mM) and incubated overnight (12 h) at 37°C and 275 rpm. The cells were collected by centrifugation ($\sim$5000$\times g$, 10 min at 4°C), washed twice with phosphate buffer, 50 mM pH 7, and disrupted by grinding with glass beads in a Bead Beater Instrument (BioSpec, Bartlesville, OK). Cell debris and glass beads were removed by centrifugation at 8,000$\times g$ for 10 min, and the supernatant was used as the cell free extract (CFE), and kept at 4°C until use.

Uroporphyrinogen III methyl transferase activity

To evaluate methyltransferase activity, assays were carrying out according to Vevódova et al. (2004) using fluorescence cuvettes (2.5 ml) containing enzyme buffer, *S*-adenosyl methionine, uroporphyrinogen III and CFE obtained as described above. Fluorescent emission was measured over 20 min: excitation 363 nm; emission 602 nm..

Expression of cobA/hemD in Lactococcus lactis

The 1.8 kb fragment corresponding to *cobA/hemD* gene of *Lb. reuteri* was released digesting the plasmid pTOPOCobA with restriction endonuclease *Sal*I. This fragment was purified and ligated to the plasmid pET28b (Novagen) digested with the same enzyme obtaining the plasmid pETCobASal.

In order to express *cobA/hemD* in *Lc. lactis*, plasmid pETCobASal was digested with restriction endonuclease *Sac*I and ligated to the lactococcal expression vector pMG36e (Dunny et al. 1991), digested with the same enzyme. Transformant colonies were tested for the presence of a 10.7 kb plasmid. One colony with a plasmid (pMGCobA) of the correct size was selected for further studies. Restriction enzymes and PCR analyses indicated that the constitutive P32 promoter of pMG36e was located immediately upstream of *cobA/hemD*. *Escherichia coli* colonies harboring this plasmid were pink under UV, which indicated that *cobA/hemD* gene was active. The recombinant plasmid (pMGCobA) was used to electro transform *Lc. lactis* NZ 9000 (Holo and Nes 1989). To test the expression of c*obA/hemD* from *Lb. reuteri* CRL 1098 in *Lc. lactis*, fluorometric assays were performed as described above. Fluorescence production by the recombinant *Lc. lactis* colonies was evaluated by growing the strain in M17 agar supplemented with uroporphyrin III (Sigma-Aldrich).

Results and discussion

Cloning and overexpression of cobA/hemD in Escherichia coli

In silico analysis of the cobalamin biosynthesis cluster from *Lb. reuteri* CRL1098 showed that an

ORF of 464 amino acids has similarity with the CobA protein of *Propionibacterium freudenreichii* that codifies for the Uroporphyrinogen III methylase (Sattler et al. 1995). In order to verify that this 464 a.a. ORF possess urogen III methylase activity, the gene was cloned in *E. coli* using the plasmid pCR TOPO. This was achieved out using specific primers designed from the sequence of cobalamin biosynthesis cluster from *Lb. reuteri* CRL1098.

Previous reports described the use of the *cob*A gene as a fluorescent transcriptional reporter and developed some vectors for use in *E. coli*, yeasts and mammalian cells (Wildt and Deuschle 1999). Over-expression of the methylase in *E. coli* results in the intracellular accumulation of sirohydrochlorin and trimethylpyrrocorphin, which emit a reddish fluorescence upon illumination with UV light (Warren et al. 1990; Wildt and Deuschle 1999). In our experiments, the colonies bearing the plasmid pTOPOCobA exhibited a pale pink fluorescence indicating that the *cobA/hemD* gene from *Lb. reuteri* CRL1098 was active in *E. coli* (data not shown).

In order to determine the orientation of the *cobA/hemD* gene in pTOPOCobA, this plasmid was analyzed by PCR using vector primers T7, M13 and the internal primers CobF and CobR. The amplification profile showed that all selected colonies carried recombinant plasmids with the *cob*A gene cloned downstream of the T7 promoter, and in opposite orientation to the *lac* promoter (data not shown), suggesting that the *cobA/hemD* gene from *Lb. reuteri* was expressed under the control of a promoter located upstream of the cloned fragment. A promoter search using the NNPP program (available at http://www. fruitfly.org/seq_tools/promoter.html) suggested a putative transcriptional promoter site with conserved −35 and −10 boxes (Fig. 1), although a recent work did not show that this promoter was active in *Lb. reuteri* (Santos et al. 2008).

Since *cobA* was cloned downstream of the T7 promoter, plasmid pTOPOCobA was isolated and used to transform strain *E. coli* BL21(DE3) which express the T7 RNA polymerase under the control of the *lac* promoter. When the transformed colonies were illuminated with UV light a strong pink fluorescence was observed in a plate with IPTG (Fig. 2a), although an important pink fluorescense was also detected in the absence of the inducer (data not shown). Although the plasmid pCR 2.1 TOPO TA

carries a *lac* promoter we did not observed growth problems with the transformed *E. coli* BL21(DE3) in the presence of IPTG.

In order to confirm that the pink fluorescence was due to the methylase activity of CobA, fluorescence assays were performed using the CFE of the recombinant *E. coli* and uroporphyrinogen III as a substrate. As shown in Fig. 3a, the *E. coli* extract of the strain harboring plasmid pTOPOCobA, showed a high fluorescence only when the substrate Urogen III was present along with the cofactor S-adenosylmethionine.

Expression of *cobA/hemD* in *Lactococcus lactis*

In order to express *cobA/hemD* in *Lc. lactis*, the insert from plasmid pTOPOCobA was subcloned in *E. coli* expression vector pET28a. This recombinant plasmid (pET-CobA) was fused to the lactococcal vector pMG36e as described in Material and methods. The recombinant plasmid harbored replication origins for *E. coli* and *Lactococcus*, and the *cobA/hemD* gene oriented in the same direction of the P32 promoter from pMG36e. This plasmid was used to transform *Lc. lactis* NZ9000. Since this strain does not possess *hem* or cobalamin genes (Linares et al. 2010) uroporphyrin III was added to the plates to verify that the colonies were able to methylate this compound and produce the pink fluorescence. When *Lc. lactis* colonies were illuminated with UV light no fluorescence was observed in the plates. One possible explanation is that the expression of the *cobA/hemD* gene under the P32 promotor is not strong enough to overproduce the fluorescent compounds to be detected by this method. However when using the CFE of one of the lactococcal *cobA/hemD* recombinant colonies, it was possible to observe a weak methylase activity in the fluorescence assay that detect the precorrin-2 and sirohydrochlorin synthesis (Fig. 3b). These results are in agreement with SDS-PAGE analysis of the CFE of *Lc. lactis* (+pMG-CobA), which showed a weak band of 51 kDa corresponding to the urogen III methylase synthase enzyme (data not shown).

Complementation of a *hemD* mutant

In silico analysis showed that CobA is part of the putative bifunctional protein corresponded to the first 240 aa of the *N*-terminus. The CobA part of the
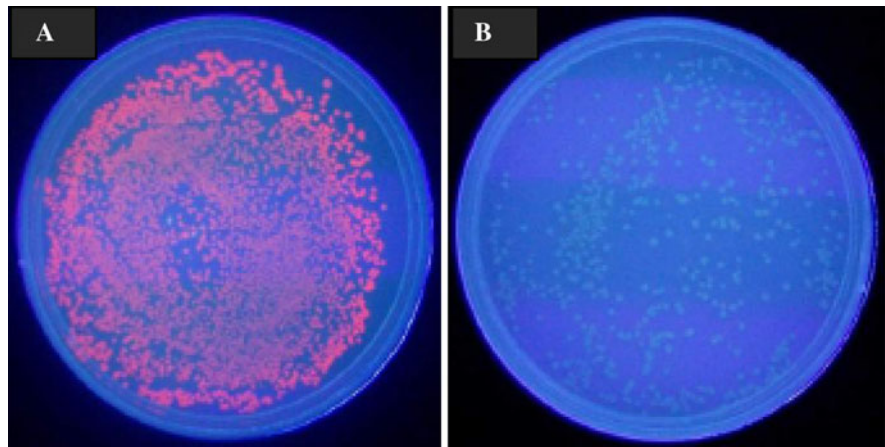
Fig. 1 Partial sequence of *cobA/hemD* gene from *Lb. reuteri* CRL 1098 and the 5′ adjacent region with possible promotor activity. Indicated in *bold* are the probable −35 and −10 sites (*in silico* analysis) and the beginning of the ORF of CobA/HemD (accession number AY780645.1)

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1    GCCAAGGTGGGGTTCAAGAAAAGATAGCTGCTTGTCAGCAATTAGGAATTCCCTGCATAA

                                 -35?                          -10?

61   TTATTCGTCGACCACAAATAAGCTATCCACATATGGTTTCGAGTTTACAGGAATTGGAGA


121  AATACTTGGAGGAAAACGATGAATGGTAGAGTAACACTTCTAGGTGCCGGACCGGGTAAT

       CobA/HemD  --->   M   N   G   R   V   T   L   L   G   A   G   P   G   N

181  CCAGAATTATTAACGTTAATTGGCAAGCGGCGATTAAATGAAGCGGATATCGTCTTGTAT

61    P   E   L   L   T   L   I   G   K   R   R   L   N   E   A   D   I   V   L   Y

241  GATCGGTTAATTGATCCTTCGTTATTAGCATTTACAAATAATGAGGCGGAATTGATCGAT

81    D   R   L   I   D   P   S   L   L   A   F   T   N   N   E   A   E   L   I   D

301  GTTGGGAAATTACCTCTACATCATAAGGTTAAGCAGTCGAAAATCAATGAAATGTTAGTT

101   V   G   K   L   P   L   H   H   K   V   K   Q   S   K   I   N   E   M   L   V
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Fig. 2 Expression of the *Lb. reuteri* CRL 1098 *cobA* gene in *E. coli*. Plates were illuminated with UV light and photographed. **a** *E. coli* BL21(DE3) transformed with pTOPOCobA. **b** Control, *E. coli* BL21(DE3) transformed with plasmid pCR TOPO without insert

protein showed high similarity (>50%) with other CobA protein form different microorganisms. However when Blast analysis were performed with the COOH terminus (aminoacids 241–461), a relatively low similarity (identity of aprox. 28%) was found with urogen III synthases from some Gram + microorganisms. To verify that CobA/HemD from *Lb. reuteri* CRL 1098 has also urogen III synthase, complementation studies were performed with the mutant strain *E. coli* SASZ31. *Eschericha coli* SASZ31 harboring the plasmid pTOPOCobA cultivated in TBAB medium without hemin and cysteine grew as small colonies; in contrast the transformation with the vector alone did not allow the growth of the *hemD* strain (data not shown). This result suggests that CobA/HemD from *Lb. reuteri* also possess Urogen III synthase activity, and therefore it could be considered a bifunctional enzyme. Similar results were obtained in *Selenomonas ruminantium,* which also possess the urogen III methylase fused to the synthase activity (Anderson et al. 2001).

In this work we report the cloning, and overexpression of the *cobA* gene from *Lb. reuteri* CRL 1098, encoding for the urogen III-methyltransferase (CobA), considered in different organisms a key enzyme in the first steps of the cobalamin and tetrapyrrol biosynthesis. In this microorganism the *cobA* gene is fused with the urogen III synthase gene (*hemD*), generating a bifunctional gene with urogen III synthase and methylase activity. As indicated
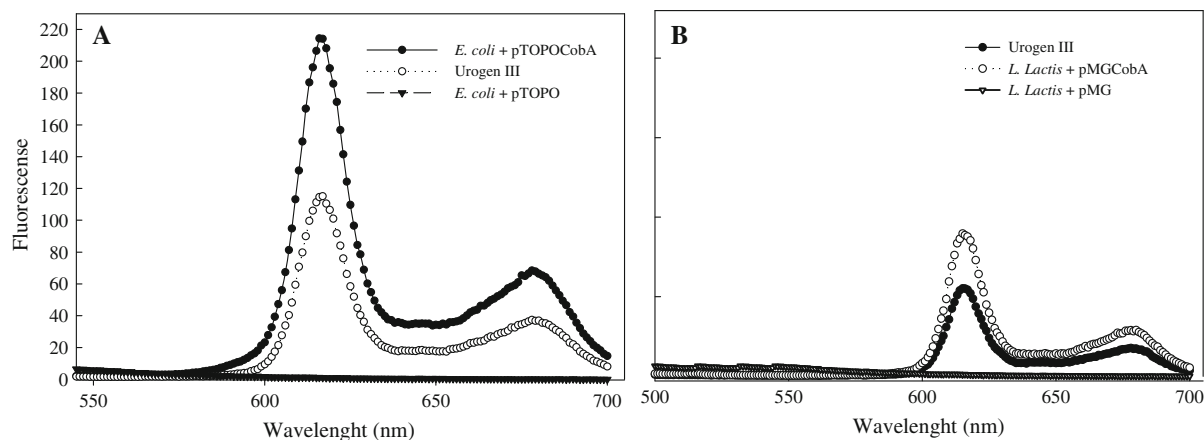
Fig. 3 Fluorescence spectroscopy emissions obtained for the cobA/hemD methylase activity of *Lb reuteri* CRL1098. **a** CFE of *E. coli* BL21 bearing pTOPOCobA plasmid, **b** CFE of *Lc. Lactis* NZ9000 bearing pMGCobA plasmid. Urogen III: emission due to the substrate alone. *E. coli and Lactococcus* transformed with the respective vectors without the cobA/hemD gene cloned were used as controls

above, the cobalamin biosynthesis cluster of *Lb. reuteri* is similar to that of *S. enterica*. However, there are some important differences: the location of *hem* genes and the presence of *cysG* in *S. enterica* that codified for a multifunctional enzyme. Since *Salmonella* is a respiratory organism, there some differences, particularly in the *hem* genes loci. In *Lb. reuteri* a heterofermentative, non respiratory organism, the *hem* genes are embedded in B_{12} biosynthesis cluster. Besides *Lb. reuteri* does not possess the last genes involved in the heme synthesis. Other important difference in the pathway is the presence of the CysG protein in *Salmonella*. CysG is a multifunctional protein that codes for siroheme synthase (Fazzio and Roth 1996). In *Salmonella* the *cobA/hemD* gene is absent (Raux et al. 1996).

According to Woodcock et al. (1998), it is possible to cluster the available sequences of cobA from a number of bacterial species in three categories: (1) In species such as *Bacillus megaterium*, *P. freudenreichii* and *P. denitrificans*, CobA is a protein of about 25–30 kDa; (2) in *E. coli, Salmonella* and related bacteria, it is a multifunctional protein of aproximately 50–55 kDa, named CysG, which is composed of a *N*-terminal domain with precorrin-2 oxidase and ferrochelatase activities involved in transforming precorrin-2 into sirohaem, and a *C*-terminal domain with *S*-adenosyl-ʟ-methionine-dependent Uroporphyrinogen III C Methyltransferase (SUMT) activity; and (3) in *Paenibacillus maceras, Clostridium josui* and

Bacillus halodurans, methylase activity is associated to a bifunctional protein (~ 55 kDa); the transmethylase activity is fused to the uroporphyrinogen III synthase (EC: 4.2.1.75), enzyme responsible of the generation of urogen III from uroporphobilinogen. Based on its size and similarity with other enzymes from the group 3, and the complementation study, the CobA/HemD protein from *Lb. reuteri* CRL 1098 could be included in this last category.

Although the overexpression of urogen III methylase was not achieved in *L. lactis*, the methylase activity could be observed by fluorometry. Since the gene is active in this food-grade bacterium, this result could open doors for the construction of new food-grade vectors based on the fluorescence generated by *Lb. reuteri* CobA activity, using more efficient promoters. For this purpose, efficient expression systems and multicopy plasmids that replicate in different LAB host will be necessary to attain high level of fluorescence detectable in single colonies. Besides the overexpression of this gene in B_{12} producer strains would be important to determine if the high expression of *hemD/cobA* leads to a higher level of cobalamin production.

In conclusion, a better knowledge of the key B_{12} biosynthesis genes and their regulations will be very important for the development of new modified strains with the ability to overproduce this essential vitamin.

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