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Pseudovitamin B_{12} is the corrinoid produced by *Lactobacillus reuteri* CRL1098 under anaerobic conditions

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Abstract **We have reported previously on the ability of *Lactobacillus reuteri* to produce a compound with vitamin B_{12} activity. Here we report on the chemical characterisation of this corrinoid-like molecule. High performance liquid chromatography coupled to an ultraviolet diode array detector, mass spectrometry and nuclear magnetic resonance spectroscopy has enabled us to identify the compound as Coα-[α-(7-adenyl)]-Coβ-cyanocobamide or pseudovitamin B_{12}. This molecule differs from cobalamin in the α-ligand, where it has adenine instead of 5,6-dimethylbenzimidazole bound in a α-glycosidic linkage to C-1 of ribose. *L. reuteri* is the first lactic acid bacterium in which the production of a cobalamin-like molecule has been identified and the first microorganism reported to produce exclusively pseudo-B_{12}.**

Keywords: B_{12} analogues; Cobamide; Pseudovitamin B_{12}; Corrinoids; Lactic acid bacteria; *Lactobacillus reuteri*

1. Introduction

Lactobacillus reuteri is a Gram-positive, heterofermentative lactic acid bacterium, frequently found in the gastrointestinal tracts of humans and other animals [1,2]. Relevant probiotic properties such as the lowering of blood cholesterol levels [3], and a direct anti-inflammatory activity [4–6] have been demonstrated for this microorganism. During growth on glucose and in the presence of glycerol, *L. reuteri* possesses the ability to produce and excrete reuterin [7] (Fig. 1). This broad spectrum antimicrobial is a mixture of monomeric, hydrated monomeric and cyclic dimeric forms of 3-hydroxypropionaldehyde (3-HPA) [8]. The synthesis of reuterin is mediated by a B_{12}-dependent enzyme, glycerol dehydratase, which catalyses the conversion of glycerol to 3-HPA [9].

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Abbreviations: 3-HPA, 3-hydroxypropionaldehyde; DMB, 5,6-dimethylbenzimidazole; UV-DAD, ultraviolet diode array detector; pseudo-B_{12}, Coα-[α-(7-adenyl)]-Coβ-cyanocobamide

We have reported, previously, that a compound isolated in its cyano form from *L. reuteri* CRL1098 was capable of fulfilling the auxotrophic B_{12} requirements of three indicator strains [10]. In the same study, DNA-sequences predicted to encode enzymes of the anaerobic B_{12} biosynthesis pathway were identified in the chromosome of *L. reuteri*.

A great variety of vitamin B_{12} analogues can be found in nature. They share a structural architecture consisting of a corrin ring with a cobalt ion chelated at the core. Cobalamin, the best studied corrinoid, is a cobamide in which 5,6-dimethylbenzimidazole (DMB) is the aglycon attached to the α-ligand bound in an α-glycosidic linkage from its N-1 to the C-1 of ribose. B_{12} biosynthesis is only found in a few prokaryotes [11]. Some have been described to synthesize B_{12} analogues that contain bases in the α-ligand other than DMB, namely other benzimidazoles, purines and phenolic compounds [12].

Here we report on the chemical characterisation of the corrinoid-like molecule isolated from *L. reuteri* in its cyanided form. Using high performance liquid chromatography (HPLC) coupled to an ultraviolet diode array detector (UV-DAD) followed by mass spectrometry and nuclear magnetic resonance spectroscopy, we have concluded that the corrinoid produced by *L. reuteri* CRL1098 cultured under anaerobic condition is pseudovitamin B_{12}. First reported in 1952 by Pfiffer et al. [13] this B_{12} analogue differs from cobalamin in the α-ligand, where DMB appears substituted by adenine. Because the majority of the structure is conserved, it has been suggested that this molecule could play a role in assessing the capability of B_{12}-dependent enzymes to utilize alternative cofactors [14], and in understanding the impact of B_{12} analogues in vitamin B_{12} metabolism [15,16].

2. Materials and methods

2.1. Preparation of cultures and cell-extracts

A culture of *L. reuteri* CRL1098 was inoculated in vitamin B_{12}-free assay medium (Difco), grown at 37 °C for 16 h and transferred three times. Two different growth conditions, with and without adding DMB (100 mg/l), were used in these studies. Cell-extracts were prepared from a 10 L batch culture flushed with a mixture of 95% N_2 and 5% CO_2, containing approximately 50 μg/L of corrinoid. After harvesting, cells were washed twice in 0.1 M phosphate buffer, pH 7.0, resuspended in 10 mL of extraction buffer consisting of 0.1 M Na_2HPO_4, pH 4.5 (citric acid) and containing 0.005% KCN. The cell suspension was separated in 10 aliquots of 1 mL each, disrupted with 1 g of glass beads (0.1 mm diameter) in a FastPrep FP120 (Qbiogene,

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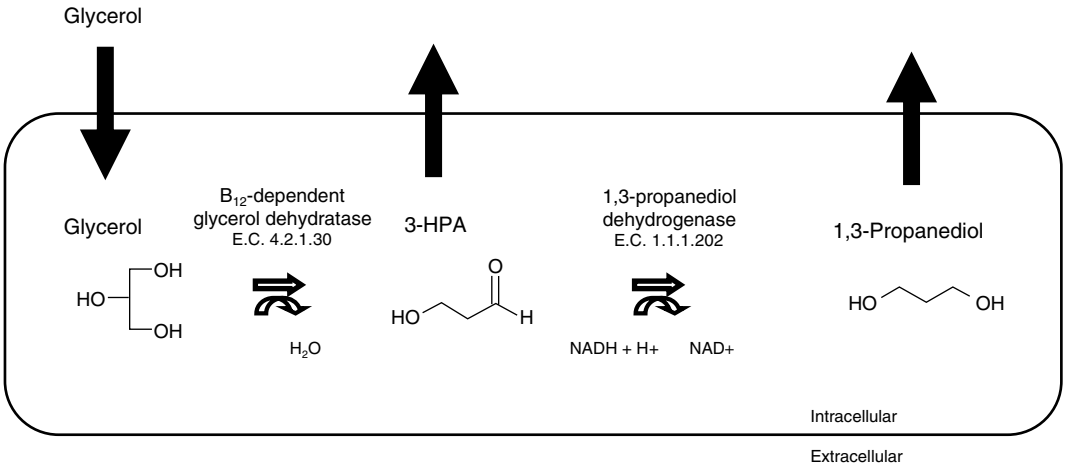

Fig. 1. Schematic representation of glycerol metabolism in *L. reuteri*.

Carlsbad, Calif.) and again combined. Extraction buffer was then added up to a final volume of 20 mL and autoclaved (120 °C for 15 min). The mixture was cleared by centrifugation ($8000 \times g$ for 10 min), and the supernatant was passed over an Isolute solid-phase extraction (SPE) column (500 mg C18 end-capped column with a 3-ml reservoir volume) previously activated with 2 ml of acetonitrile. The column was washed twice with 2 volumes of distilled water to remove salts and other hydrophilic contaminants. Subsequently, the corrinoid was eluted with 1 volume of 50% acetonitrile and concentrated to dryness in vacuo at 30 °C. The residue was dissolved in 5 ml of sterile distilled water and stored in the dark at −20 °C until further use.

2.2. Corrinoid isolation

The corrinoid was purified from cell-extracts of *L. reuteri* by reverse-phase high performance liquid chromatography (RP-HPLC) with a Waters (Milford, MA) 600E system automated gradient controller, a 250- by 3-mm Betasil phenyl column (Thermo Hypersil–Keystone, Waltham, MA), and a SPD-M10A VP Diode Array Detector (Shimadzu Corporation, Kyoto, Japan). The fraction showing a UV-DAD spectrum similar to that of cyanocobalamin was collected manually and lyophilised at 30 °C.

2.3. Mass spectrometry

The sample collected from the RP-HPLC was dissolved in 100 μl of an aqueous solution containing 30% acetonitrile and 0.1% formic acid. Mass spectrometric analyses of the corrinoid purified from *L. reuteri* were performed on a Quatro II triple-quadrupole mass spectrometer (Micromass, Inc., Altrincham, UK) equipped with an electrospray ionization (ESI) probe, operated in the positive ion mode. Experiments were carried out under the following conditions: capillary voltage 4.2 kV, cone voltage 36 V, extraction voltage 5 V, source temperature 80 °C, desolvation temperature 120 °C.

2.4. Nuclear magnetic resonance spectroscopy

Proton nuclear magnetic resonance (^{1}H NMR) spectra were acquired on a Bruker DRX500 spectrometer (Bruker, Rheinstetten, Germany) using a 5 mm inverse-detection probe head. The sample purified from the RP-HPLC was lyophilised at 30 °C and dissolved in 10% D_2O, which allows the detection of amide resonances. Spectra were recorded at 303 K with pre-saturation of the water signal, using an 8.25 s pulse width corresponding to a 75° flip angle and a repetition delay of 3 s.

3. Results

During purification, UV-DAD spectra data and retention times obtained by RP-HPLC analyses showed that *L. reuteri* CRL1098 grown under anaerobic conditions produces one major cobalamin-like molecule, in a ratio of $\geqslant$100:1 in relation to other minor corrinoid species. The identification and characterisation of the major cobamide produced by this strain was based on the application of three different techniques: RP-HPLC isolation coupled to UV-DAD spectrum determination, mass spectrometry analysis and NMR spectroscopy studies.

3.1. Corrinoid isolation

To isolate and purify the native corrinoid produced by *L. reuteri*, cell-extracts were eluted on a C18-SPE column followed by RP-HPLC. The cell-extracts from *L. reuteri* revealed a peak with a retention time of 36.31 min (Fig. 2A). Although the peak displayed similar UV-DAD spectra, this retention time did not agree with the one of the cyanocobalamin standard (37.83 min). The spectrum of the standard showed a peak of maximal absorbance at 350 nm and two other at 512 and 550 nm, respectively. The same pattern was observed when the isolated cobamide produced by *L. reuteri* was analyzed (Fig. 2B). Identical chromatography patterns and UV-spectra were recorded for the corrinoids isolated from *L. reuteri* CRL1098 grown in the presence or absence of DMB.

3.2. Mass spectrometry

The mass spectrometry data (Fig. 3) obtained for the corrinoid produced by *L. reuteri* provided evidence that the complete molecule has a very similar *m/z* value to the one of methylcobalamin, 1344.6 and 1344.8, respectively. In both the mass spectra of the isolated compound and of the standard, the peaks corresponding to the displacement of both the β-ligand along with the ribosyl-bound aglycon (*m/z* 1183.6) and cobinamide (*m/z* 971.6) were identified. The presence of such peaks in the mass spectrum of our sample is highly suggestive that it is indeed a B_{12} analogue.

However, when all signals displayed on the mass spectra were considered in detail, the fractions in which only the upper ligand was not present, revealed a mass variation of 15.1 for the methylcobalamin standard (*m/z* 1329.7) and 26.0 for the isolated compound (*m/z* 1318.6), corresponding to the displacement of a methyl and a cyano radical, respectively. This implied that the α-ligand of methylcobalamin and the isolated compound differed approximately 11 mass units.