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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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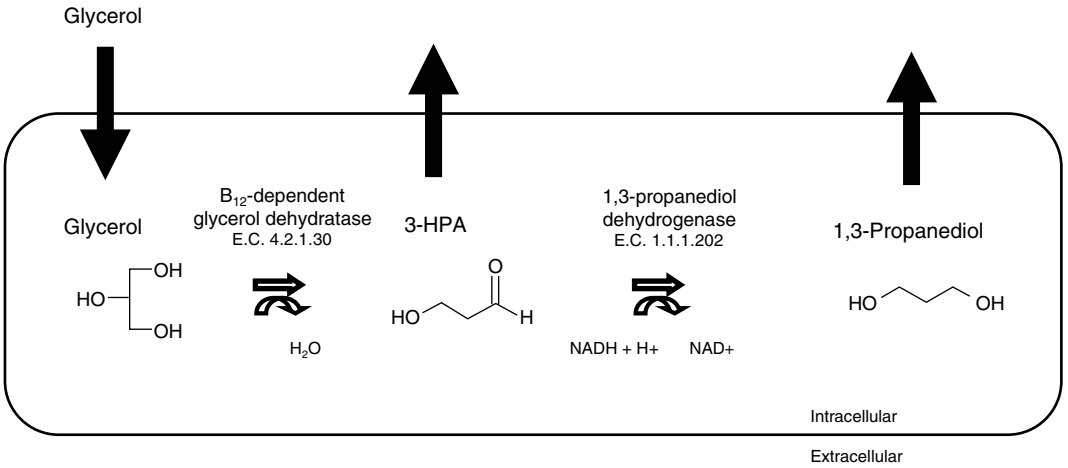

Fig. 1. Schematic representation of glycerol metabolism in *L. reuteri*.

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.

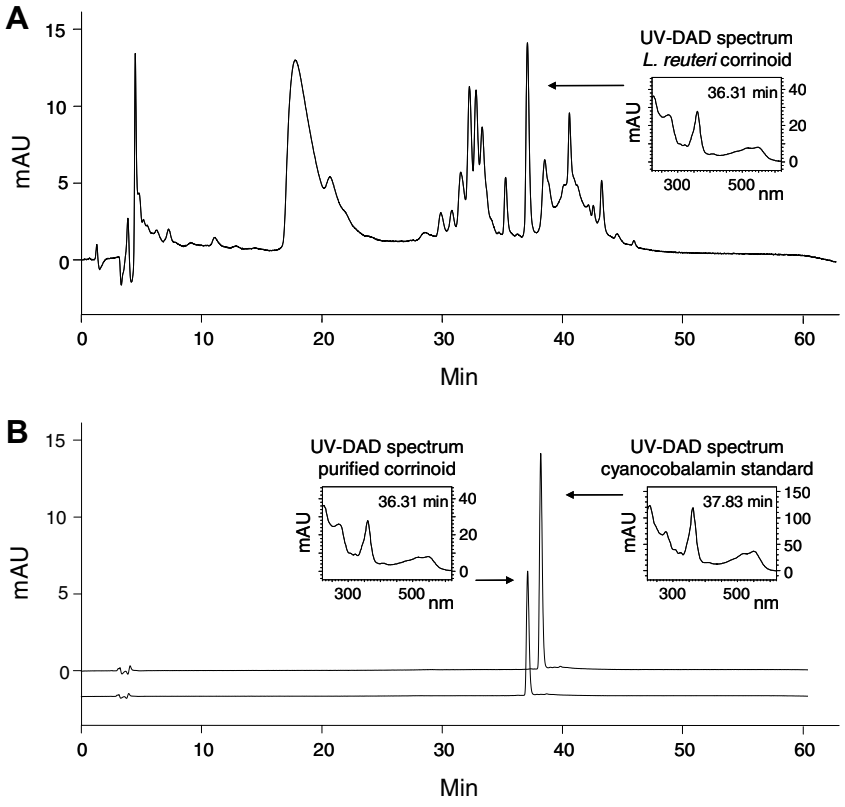

Fig. 2. (A) HPLC chromatogram of the cell-extract and UV-DAD spectrum (inset) of the corrinoid produced by *L. reuteri* under anaerobic conditions in the presence of DMB. The same pattern was observed in the absence of DMB. (B) HPLC chromatograms and UV-DAD spectra (inset) of the purified corrinoid produced from *L. reuteri* CRL1098 and the cyanocobalamin standard. Although their retention time does not coincide, both the spectra of the corrinoid purified from *L. reuteri* and the cyanocobalamin standard, display a peak of maximal absorbance at 350 nm and two other at 512 and 550 nm, respectively.

A peak with an *m*/*z* value of 359.3 was identified in the methylcobalamin standard, but not in the corrinoid purified from *L. reuteri*. This peak is derived from the lower ligand in which DMB is the aglycon attached by a glycosyl bound from its N-1 to the C-1 of ribofuranose 3-phosphate. Instead, another peak is present in the mass spectrum of the corrinoid purified from *L. reuteri* with an *m*/*z* value of 348.2, once again implying a mass variation of approximately 11 in the α-ligand. Furthermore, the peaks with an *m*/*z* value of 456.9 and 486.5, corresponding to different fractionation in which the corrin ring and the upper ligand are displaced (Fig. 4A), could be identified in the methylcobalamin standard but not in the corrinoid purified from *L. reuteri*. Hence, the possibility that the compound produced by *L. reuteri* had DMB in the lower ligand was ruled out.

The mass spectrum recorded for the isolated compound showed a peak corresponding to the sole displacement of the cyano radical (*m*/*z* 1318.6), which was in accordance with the substitution in the lower ligand of the DMB moiety by adenine. Also the observed peak with an *m*/*z* value of 348.2 corroborated this substitution, and thus, explained the difference of 11 mass units mentioned above. This observation implied that the corrinoid extracted from *L. reuteri* was actually Coα-[α-(7-adenyl)]-Coβ-cyanocobamide, commonly known as pseudo-B_{12}. Furthermore, the observed peaks in the spectrum with *m*/*z* values of 433.3 and 457.1 were consistent with this proposed structure and represent different fragmenting of the lower ligand containing adenine instead of DMB (Fig. 4B).

3.3. Nuclear magnetic resonance spectroscopy

Additionally, a ^{1}H NMR spectrum taken of the corrinoid purified from *L. reuteri* (Fig. 5) showed striking similarities with published NMR spectra of pseudovitamin B_{12}. In fact, a comparison between the acquired signals and those obtained for pseudovitamin B_{12} by Hoffman et al. [17] showed only six signals that were not present in the published spectrum. However, these signals are also not present in the spectra of other corrinoids, and therefore, we have assumed they are caused either by minor contaminants (peaks marked 2, 4 and 5 in Fig. 5), or by substances used during sample preparation (peaks marked 1, 3 and 6 in Fig. 5). Furthermore, the presence in the ^{1}H NMR spectrum of a characteristic sharp signal around 8 ppm is indicative of a highly unshielded aromatic proton in its structure, which, by itself, rules out the identification of the corrinoid as cobalamin or factor A, both molecules lacking such a proton. Pseudovitamin B_{12}, on the other hand, has such a proton in its structure.

A ^{1}H homonuclear correlation spectrum was recorded and showed no visible correlations to the three signals at lower-field (8–9 ppm) (data not shown). This is in agreement with the proposed structure for the α-ligand of the native corrinoid of *L. reuteri*, and dismisses the possibility of the presence of [N8-methyl]-adeninyl-cobamide.

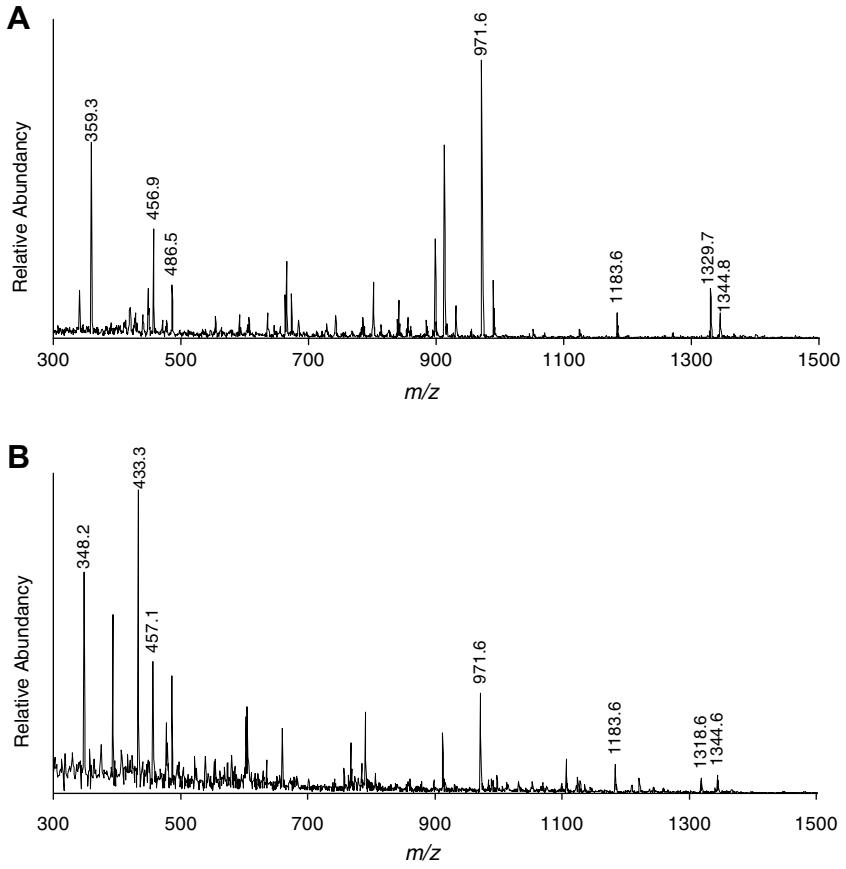

Fig. 3. (A) Mass spectrometry spectrum of methylcobalamin. The peak with an *m/z* value of 1344.8 corresponds to the intact molecule; 1329.7 corresponds to the displacement of the upper ligand, the methyl radical; 1183.6 corresponds to the displacement of both the upper ligand and DMB; 971.6 corresponds to cobinamide; 359.3 corresponds to the lower ligand in which DMB is the aglycon attached to ribofuranose 3-phosphate. The peaks with an *m/z* value of 486.5 and 456.9 correspond to different fractioning in which both the corrin ring and the upper ligand have been displaced. (B) Mass spectrometry spectrum of the corrinoid purified from *L. reuteri*. The peak with an *m/z* value of 1344.6 corresponds to the intact molecule; 1318.6 corresponds to the displacement of the upper ligand, a cyano radical; 1183.6 corresponds to the displacement of both the upper ligand and adenine; 971.6 corresponds to cobinamide; 348.2 corresponds to the lower ligand in which adenine is the aglycon attached to ribofuranose 3-phosphate. The peaks with an *m/z* value of 457.1 and 433.3 correspond to different fractioning in which both the corrin ring and the upper ligand have been displaced.

4. Discussion

The corrinoid extracted by *L. reuteri* under anaerobic conditions has been identified as Coα-[α-(7-adenyl)]-Coβ-cyanocobamide, commonly known as pseudovitamin B_{12}. The results obtained performing mass spectrometry and nuclear magnetic resonance spectroscopy enabled us to identify the corrin ring with an atom of cobalt at the core, a cyano radical as the β-ligand, and adenine as the aglycon bound by a glycosyl link from its N-1 to the C-1 of the ribose forming the α-ligand.

During the purification of the corrinoid produced by *L. reuteri*, the difference in retention time between our sample and the cyanocobalamin standard was suggested to be due to a difference in the phosphorylation state of the molecule. This was supported by a previous report on the *in vitro* synthesis of the cobalamin nucleotide loop [18], in which, the compound was identified as cyanocobalamin-5′-phosphate using mass spectrometry. Based on this, we collected the purified molecule with the same UV-DAD spectrum as cyanocobalamin and performed mass spectrometry analysis for molecular characterisation. Considering all the cobalamin analogues known [12], pseudovitamin B_{12} was the only one with full spectral consistency based on the mass spectra collected. This analogue has a structure similar to that of cobalamin except for the lower ligand, in which, the DMB moiety is substituted by adenine. The mass spectrometry results reported here enabled the identification of several peaks corresponding to the fractioning of the lower ligand containing an adenine base (Fig. 3B). Additional NMR studies were performed to confirm the identity of the corrinoid. The conjunction of all the spectral information acquired makes it clear that the corrinoid extracted from *L. reuteri* was indeed pseudo-B_{12}.

Recently, the gene *bluB* has been shown to be necessary for the aerobic biosynthesis of DMB in *Sinorhizobium meliloti* [19]. It is involved in the fragmentation and contraction of flavin mononucleotide to form D-erythrose 4-phosphate and DMB in the presence of oxygen. The recently released genome sequence of *L. reuteri* JCM1112 (DOE Joint Genome Institute, GeneBank Accession no. CP000705) lacks a homologue of *bluB* and the growth of this lactic acid bacterium is impaired in the presence of oxygen. As a consequence, we consider the presence of such pathway in *L. reuteri* to be unlikely. *Salmonella enterica* has been reported to be able to incorporate exog-

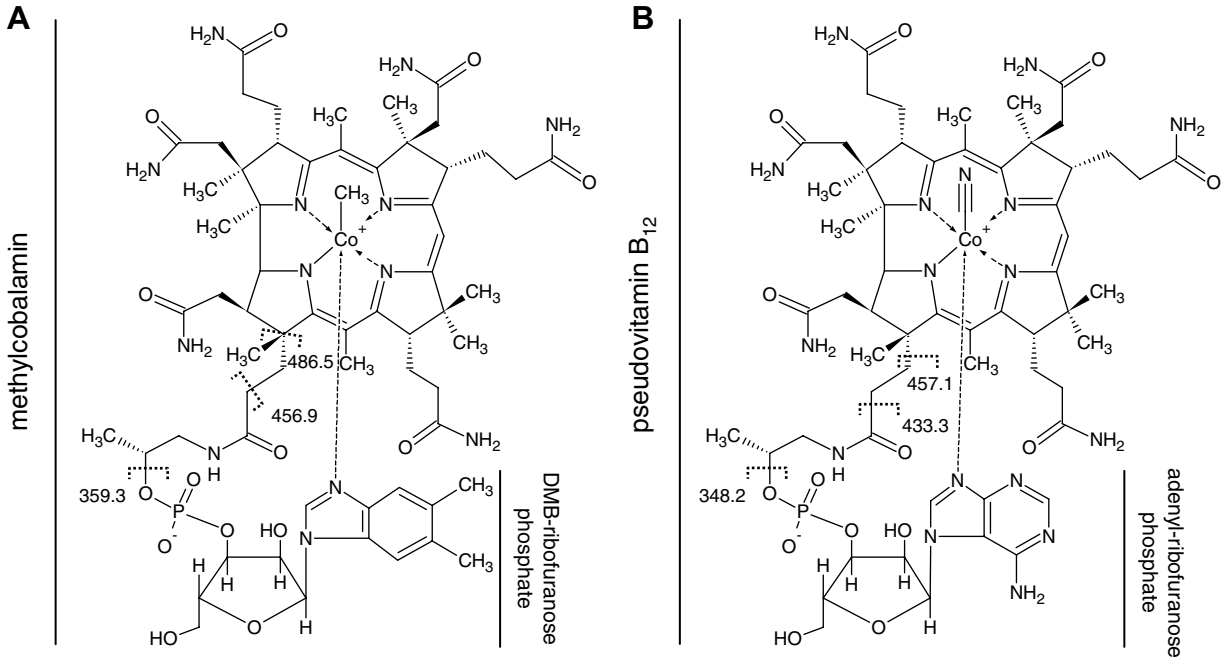

Fig. 4. Chemical structure of (A) methylcobalamin and (B) pseudovitamin B_{12}: likely structures and measured *m/z* values of different fractions of the lower ligand.

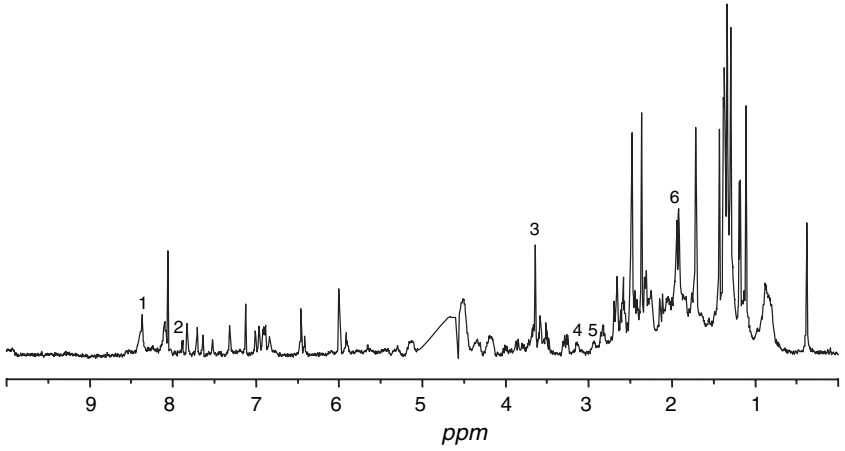

Fig. 5. Proton nuclear magnetic resonance spectrum of the corrinoid purified from *L. reuteri*. ^{1}H NMR spectrum was acquired on a Bruker DRX500 spectrometer using a 5 mm inverse-detection probe head and recorded with presaturation of the water signal, using an 8.25 s pulse width corresponding to a 75° flip angle and a repetition delay of 3 s. The sample was dissolved in 10% D_2O. Peaks marked 1–6 have been assigned to contaminants.

enous DMB into the lower ligand producing cobalamin [20]. We could not detect a similar behaviour for *L. reuteri* CRL1098, which is not completely unexpected. Both phenotypically and phylogenetically, these two species are quite different, and thus, it is not surprising that they have different uptake capacities.

Some genera of cyanobacteria have been reported to contain pseudo-B_{12} amongst other corrinoids [21,22]. The same applies to *Clostridium cochlearium* [17], another Gram-positive bacterium with a low G+C content in its DNA. Interestingly, *L. reuteri* is the first microorganism reported to biosynthesize pseudovitamin B_{12} as its sole corrinoid product. Pseudo-B_{12} is not commercially available and until now its supply has solely relied on guided biosynthesis or chemical synthesis [23–25].

Vitamin B_{12} and several of its analogues act as cofactors, catalyzing methyl-transfer and carbon-backbone rearrangement reactions. The comparison of the cofactor efficacy of the different analogues enables the elucidation of complex enzyme–cofactor interactions. Future studies of the capability of B_{12}-dependent enzymes to use pseudo-B_{12} as a cofactor, may rely on *L. reuteri* for its provision. Ultimately, this will help in the effort to elucidate the relevance of the lower ligand and Co–N coordination for enzymatic activity.

In humans, *in vitro* studies on B_{12} binding proteins have shown that only intrinsic factor can present a real bottleneck for the uptake of pseudo-B_{12} [25]. However, comparative *in vivo* studies establishing the efficacy of B_{12} analogues with α-ligands other than DMB to correct B_{12} deficiency remain

to be carried out. *L. reuteri* is the first lactic acid bacterium reported to produce a cobalamin-like molecule. Besides the system of producing pseudovitamin B_{12} characterised here, the possibility to engineer the production of other corrinoids is currently under investigation. *L. reuteri* possesses the GRAS status (Generally Regarded As Safe), and therefore, could eventually be used to increase the nutritional value of fermented food and feed products.

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