Appl Microbiol Biotechnol (2002) 58:275–285
DOI 10.1007/s00253-001-0902-7

MINI-REVIEW

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Microbial production of vitamin B_{12}

Received: 7 September 2001 / Revised: 8 November 2001 / Accepted: 11 November 2001 / Published online: 20 December 2001

Abstract One of the most alluring and fascinating molecules in the world of science and medicine is vitamin B_{12} (cobalamin), which was originally discovered as the anti pernicious anemia factor and whose enigmatic complex structure is matched only by the beguiling chemistry that it mediates. The biosynthesis of this essential nutrient is intricate, involved and, remarkably, confined to certain members of the prokaryotic world, seemingly never have to have made the eukaryotic transition. In humans, the vitamin is required in trace amounts (approximately 1 µg/day) to assist the actions of only two enzymes, methionine synthase and (R)-methylmalonyl-CoA mutase; yet commercially more than 10 t of B_{12} are produced each year from a number of bacterial species. The rich scientific history of vitamin B_{12} research, its biological functions and the pathways employed by bacteria for its de novo synthesis are described. Current strategies for the improvement of vitamin B_{12} production using modern biotechnological techniques are outlined.

Vitamin B_{12} – a historical overview

Vitamin B_{12} came to prominence in the scientific world in the early 1920s, when two American physicians, Minot and Murphy, demonstrated that they were able to cure pernicious anemia, a disorder first described in 1835, with a diet that included whole liver. Their discovery initiated investigations into identifying their so-called "extrinsic factor", giving rise to a whole new scientific research field that has culminated in a number of Nobel Prizes, awarded most notably to Minot and Murphy (together with Whipple) in 1934 for their discoveries concerning liver therapy in cases of anemia and to Hodgkin in 1964 for her determinations by X-ray techniques of the structures of important biochemical substances.

The identity of the extrinsic factor remained elusive for the next 20 years, until research groups at two leading pharmaceutical companies, one led by Folkers at Merck in the USA and the other by Smith at Glaxo in the UK, simultaneously isolated a red crystalline compound from liver that was found to defeat pernicious anemia and was designated vitamin B_{12} (Rickes et al. 1948a). Shortly thereafter, vitamin B_{12} was also found in milk powder, in beef extract and in culture broths of various bacterial genera (Rickes et al. 1948b).

The isolation of vitamin B_{12} instigated research on the structure of the anti-pernicious anemia factor and it quickly became clear that vitamin B_{12} was structurally much more complex than anything that had previously been deduced. After Barker et al. (1958) had discovered and crystallized the first biologically active coenzyme forms of pseudo-vitamin B_{12} and vitamin B_{12}, it took the pioneering and outstanding work of Hodgkin and her group to deduce the three-dimensional structure of vitamin B_{12} (cyanocobalamin) and, 5 years later, of coenzyme B_{12} (adenosylcobalamin) from crystallographic data (Hodgkin et al. 1955, 1956, 1957; Lenhert and Hodgkin 1961).

It was around the same time that a second biologically active form of vitamin B_{12} was discovered. Using ^{14}C-enriched methylcobalamin (MeCbl), which had been produced by Smith and coworkers, it was demonstrated that this could act as a cofactor for methionine synthase (Guest et al. 1962). Since then, several adenosylcobalamin-dependent and MeCbl-dependent enzymes have been isolated and identified; and much progress has been achieved in the understanding of the mechanisms and stereochemistry of B_{12}-dependent rearrangements.

During the past few years, the crystal structures of methionine synthase (Drennan et al. 1994; Dixon et al.

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1996) and methylmalonyl CoA mutase (Mancia et al. 1996) have been solved. These structures, in combination with electron paramagnetic resonance data, not only of these enzymes but also of other corrinoid-dependent enzymes, identified a subclass of corrinoid-dependent enzymes, which is characterized by the replacement of the 5,6-dimethylbenzimidazole (DMBI) moiety within the active cleft of the enzyme by a histidine residue of the protein (termed "base off"; Stupperich et al. 1990; Zelder et al. 1995; Harms and Thauer 1998). To date, additionally, the three-dimensional structures of two other B_{12}-dependent enzymes in bacteria, glutamate mutase and diol dehydratase, have been solved, the latter containing the "base on" form of the coenzyme (Reitzer et al. 1999; Shibata et al. 1999).

Besides these biochemical approaches aimed at investigating and understanding the functional complexity of vitamin B_{12}, one also has to applaud the brilliant total chemical synthesis of vitamin B_{12} achieved by Woodward and Eschenmoser in the 1960s and 1970s, with the participation of more than 100 scientists during a period of 11 years. This chemical synthesis was eventually matched by the elucidation of the biochemical pathway for cobalamin in the aerobic bacterium *Pseudomonas denitrificans* in 1993. The description of the oxygen-dependent pathway was made possible by the pioneering molecular genetics and biochemistry employed by Blanche and coworkers at Rhône-Poulenc Rorer (RPR), tethered with the chemical intuition and expertise of Battersby at Cambridge in England and Scott in Texas (Battersby 1994; Blanche et al. 1995;Scott 1998a; Thibaut et al. 1998). Evident from this research was the fact that an alternative "anaerobic" cobalamin biosynthetic pathway must exist; and this was proved to be the case, although it still remains comparatively poorly understood.

Vitamin B_{12} – structure

Vitamin B_{12} is used to describe compounds of the cobalt corrinoid family, in particular those of the cobalamin group. The final products occurring in nature from vitamin B_{12}-biosynthesis are 5′-deoxyadenosylcobalamin (coenzyme B_{12}) and MeCbl, while vitamin B_{12} is by definition cyanocobalamin (CNCbl), which represents the form mainly manufactured by industry (Fig. 1). The CN group is a result of the extraction procedure by which the compound is removed from bacterial cultures.

Coenzyme B_{12} has a molecular mass of 1,580 Da. The vitamin B_{12} molecule can be considered in three parts: a central corrin ring which contains four ligands for the central cobalt ion, a lower (alpha) ligand donated by the DMBI and an upper (beta) ligand made from either an adenosyl group or a methyl group. One should notice that, in several anaerobic bacteria, the alpha ligand is replaced by adenine, different bases or even no alpha ligand to form pseudo-coenzyme B_{12} and other equally active coenzyme derivatives. The central structural component of vitamin B_{12} is the planar corrin ring composed of four pyrrole units, which differs from the porphyrins and chlorins, (hemes, chlorophylls) by the missing methine bridge between the partially hydrogenated pyrrole rings A and D. Further differences between the corrin ring and other tetrapyrrole macrocycles are the number and type of side chains, the oxidation state of the macrocycle and the centrally chelated metal ion. The Co(III) ion in vitamin B_{12} is complexed by the four nitrogen atoms of the four pyrrole-derived rings. The fifth ligand of the Co(III) coordination sphere is supplied by the N7-atom of the DMBI, which is linked by its phosphate group to an aminopropanol group covalently linked to the propionic acid side chain of ring D.

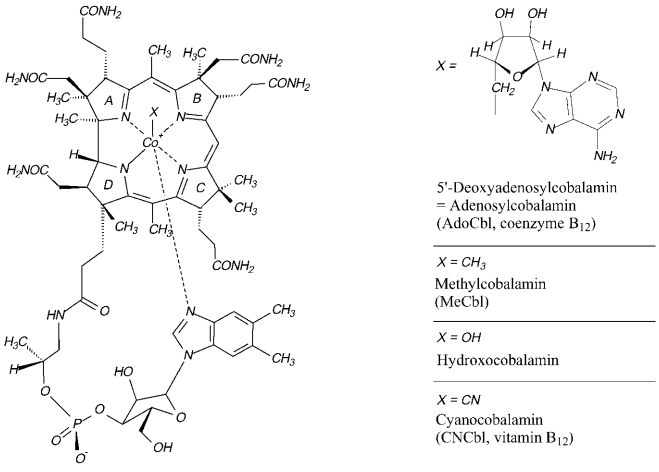

Fig. 1 The term vitamin B_{12} is widely used to describe compounds of the cobalamin group. Natural forms are adenosylcobalamin, methylcobalamin and hydroxocobalamin. Cyanocobalamin, by definition vitamin B_{12}, is the industrially produced stable cobalamin form which is not be found in nature (Hodgkin et al. 1955, 1956)

The octahedral coordination sphere of the Co(III) ion is completed by the C(5′)-atom of a 5′-deoxyadenosyl. The C-Co linkage formed was the first metal–carbon bond in biological molecules to be described. This bond is relatively weak, with a dissociation energy of about 130 kJ/mol and can therefore easily split (Finke 1998). Most of the time, the bond splitting is homolytic, in contrast to what is observed in other biological systems. Two radicals are generated as a consequence of homolysis. They provide the chemical basis for the catalytic functions of the various vitamin B_{12} derivatives. The MeCbl and 5′-deoxyadenosylcobalamin compounds are known to be sensitive to light treatment in their isolated forms and are easily transformed to hydroxocobalamin (HOCbl) at room temperature in aqueous solution (although the HOCbl remains light sensitive; Spalla et al. 1989). In the presence of cyanide the more stable CNCbl is generated.

Distribution of vitamin B_{12} – biosynthesis and utilization among living forms

What is unique about vitamin B_{12} amongst all the vitamins is that its de novo synthesis would appear to be restricted solely to some bacteria and archaea. Interestingly, animals (including humans) and protists require cobalamin but apparently do not synthesize it, whereas plants and fungi are thought to neither synthesize nor use it (Duda et al. 1967). Reports of vitamin B_{12} in plants, fungi or yeast are few and inconsistent, suggesting that they may be due to bacterial contamination (Peston 1977; Watanabe et al. 1991, 1993). It has been suggested that the distribution of vitamin B_{12} among living forms results from an evolutionary selection pressure; and this will be discussed later in this review.

Classification of vitamin B_{12}-dependent reactions

As already mentioned, there are two forms of vitamin B_{12} contributing to its biological catalytic activity: adenosylcobalamin and MeCbl. The enzymatic reactions depending on these cobalamins can be classified into three general groups (Stroinsky and Schneider 1987):

1. Intramolecular rearrangements involving the transfer of a hydrogen atom from one carbon atom to an adjacent carbon atom and its replacement by another adjacent group, catalyzed by adenosylcobalamin
2. Reduction of ribonucleotide triphosphate to 2′-deoxyribonucleotide triphosphate, catalyzed by adenosylcobalamin
3. Intermolecular methyl transfer, catalyzed by MeCbl

Some prominent vitamin B_{12}-dependent reactions in bacteria and archaea

Acetyl-CoA synthesis

The process of acetate formation in acetogenic bacteria via the Wood/Ljungdahl pathway, where acetyl-CoA is synthesized from two molecules of CO_2 is dependent on methyl corrinoids (Wood et al. 1986; Ragsdale 1991; Stupperich 1993). They mediate the methyl transfer from methyltetrahydrofolate to CO dehydrogenase, an enzyme able to bind CO by its active-site nickel. The methyl group is transferred to the CO dehydrogenase via a methyl-corrinoid/iron-sulfur protein. The enzyme uses this methyl group to synthesize acetyl-CoA from its nickel-bound CO and coenzyme A (Ferry 1995). Subsequently, the acetyl-CoA undergoes phosphorolysis to acetylphosphate, followed by phosphate transfer to ADP under the formation of ATP and acetic acid (Ljungdahl and Wood 1982; Ragsdale 1991).

Methyl transfer in methane-producing archaea

In the strictly anaerobic methane-producing archaea, methyl-corrinoids are required for the transfer of methyl groups from methanogenic substrates, like methanol (Keltjens and Vogels 1993), methylamines (Burke and Krzycki 1995) and acetate (Ferry 1992), to a thiol group of coenzyme M. (Ferry 1993; Stupperich 1993). Besides the utilization of the various methanogenic substrates as methyl donors, a methyl group can alternatively be transferred from methyltetrahydromethanopterin (a natural structural and functional analog of methyltetrahydrofolate) via MeCbl to a thiol group of coenzyme M (Poirot et al. 1987; Weiss and Thauer 1993). The considerable high energy release of approximately –30 kJ/mol (Müller et al. 1993) is recovered by coupling the methyl transfer to extrusion of sodium ions, eventually leading to a proton motive force (Becher et al. 1992; Blaut et al. 1992).

Ribonucleotide reductase

Ribonucleotide reductases play a key role in the cellular metabolism of some micro-organisms, since they generate deoxyribonucleotides from ribonucleotides, which are required for DNA synthesis. Ribonucleotide reductases can be divided into four classes, which is unusual for enzymes involved in such a central metabolic step (Jordan and Reichard 1998). The adenosylcobalamin-dependent reductases, which are primarily found in micro-organisms, belong to class II and serve as a free-radical generator, essential for the radical-based reduction process outlined in detail elsewhere (Blakely and Barker 1964; Blakely 1965; Reichard 1993; Frey 2001).

Vitamin B_{12}-dependent fermentation processes in enteric bacteria

In almost every enteric bacterium, with the exception of *Escherichia coli*, coenzyme B_{12} is essential for the anaerobic fermentation of 1,2-propanediol, ethanolamine and glycerol. During the first step of these fermentation processes, the substrates are converted to the corresponding aldehydes (Fig. 2) by an intramolecular rearrangement. These internal redox reactions are mediated by the adenosylcobalamin-dependent enzymes diol dehydratase, ethanolamine ammonia-lyase and glycerol dehydratase (Abeles and Lee 1961; Bradbeer 1965; Scarlett and Turner 1976; Toraya et al. 1979; Forage and Foster 1982; Lawrence and Roth 1995). In some bacteria, the subsequently generated propionaldehyde and the acetaldehyde serve as carbon and energy sources via oxidation to propionyl-CoA and acetyl-CoA, respectively (Obradors et al. 1988). The problem of removing the reducing equivalents resulting from these oxidation processes is solved by the reduction of the other part of the aldehydes to the corresponding alcohols, which are then excreted. In a similar fashion, glycerol dehydratase oxidizes glyc-

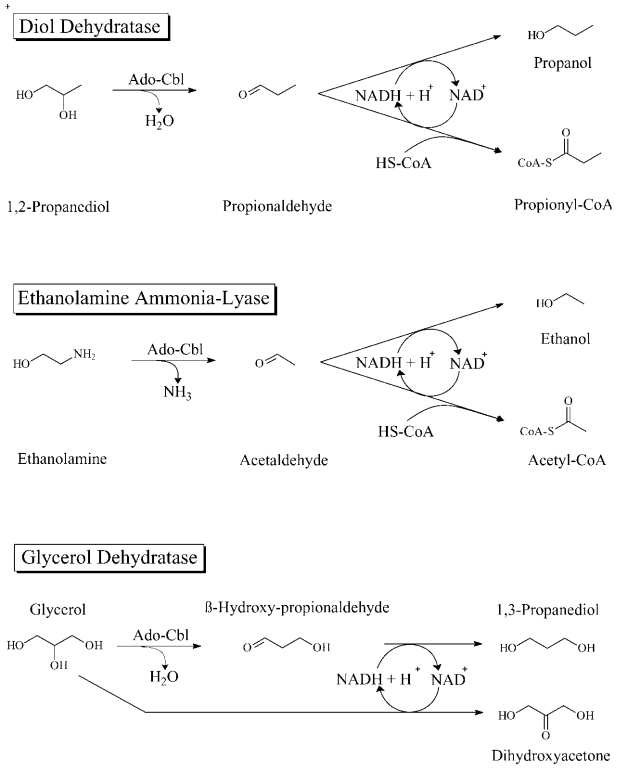

Fig. 2 Adenosylcobalamin (*Ado-Cbl*)-dependent reactions known in bacteria. The scheme outlines the steps required for generating energy, carbon-metabolites and the regeneration of reducing equivalents

erol to β-hydroxypropionaldehyde, which gets subsequently reduced to 1,3-propanediol, thereby balancing the reducing equivalents generated by glycerol dehydrogenase (Roth et al. 1996).

Vitamin B_{12}-dependent processes in humans

Animals and humans require vitamin B_{12} for only two enzymes, which are not restricted to them: (R)-methylmalonyl-CoA mutase and methionine synthase (Fig. 3). (R)-Methylmalonyl-CoA mutase is involved in the metabolism of propionyl-CoA, where the propionyl-CoA is derived from the degradation of compounds like thymine, valine, methionine and odd-chain fatty acids. In this process, propionyl-CoA is carboxylated to form (S)-methylmalonyl-CoA and is subsequently epimerized to the (R)-isomer. After rearrangement by the adenosylcobalamin-dependent (R)-methylmalonyl-CoA mutase to succinyl-CoA, it ends up in the tricarboxylic acid cycle. In vitamin B_{12}-deficient patients, the methylmalonyl-CoA intermediate accumulates and the D-isomer is cleaved by a hydrolase to coenzyme A and methylmalonic acid, which leads to acidosis. In parallel, propionyl-CoA levels increase and citrate synthase condenses propionyl-CoA with oxaloacetic acid to 2-methylcitric acid (Stabler 1999), which is a proposed inhibitor of aconitase (Van Rooyen et al. 1994).

The MeCbl-dependent enzyme, methionine synthase, methylates homocysteine to form methionine, utilizing 5-methyltetrahydrofolate as a methyl donor. Nevertheless, normal methionine concentrations are maintained in vitamin B_{12}-deficient patients. A possible explanation for this is an increased activity of cobalamin- and folate-independent betaine-homocysteine methyltransferase, which synthesizes methionine via methyl transfer from betaine to homocysteine, complemented by dietary intake of methionine (Stabler et al. 1993). One hypothesis for the cause of diseases like megaloblastic anemia argues that missing methionine synthase activity in vitamin B_{12}-deficient patients leads to the accumulation of methyltetrahydrofolate and decreased tetrahydrofolate levels, resulting in a diminished availability of folates for DNA synthesis, the so-called methylfolate trap.

As with vitamin B_6 and folic acid deficiency, vitamin B_{12} deficiency leads to an increase in homocysteine levels and consequently represents a major risk for heart disease, stroke, atherosclerosis, vascular disease etc., especially in the elderly who are particularly prone to these deficiencies (Joosten et al. 1993). At least in the case of vitamin B_{12}, this is due to an increasing malabsorption from food (Allen and Casterline 1994; Lindenbaum et al. 1994). Elevated homocysteine levels are markedly reduced by folic acid and vitamin B_{12} supplementation (Bronstrup et al. 1998).

In contrast, no solid scientific explanation is available for the importance of vitamin B_{12} in maintaining normal myelination of nerve cells. Nevertheless, it is becoming clearer that many neurological and psychiatric symptoms like ataxia, spasticity, muscle weakness, dementia, psychoses, Alzheimer's disease etc. may also be linked to vitamin B_{12} deficiency (McCaddon and Kelly 1994; Delva 1997).

Pernicious anemia, the once fatal disease that essentially led to the discovery of vitamin B_{12}, is now thought to be a gastric autoimmune disease, in which antibodies develop to intrinsic factor, which is essential for vitamin B_{12} uptake in the stomach (Stabler 1999). Since pernicious anemia results in an insufficient uptake of vitamin B_{12} from the diet, it is a common strategy to supply patients with vitamin B_{12}, to overcome the dietary shortage.

Finally, due to the high binding capacity of the central cobalt ion to cyanide, HOCbl is well known as an efficacious, safe and easily administered cyanide antidote, which is regarded as ideal for out-of-hospital use in suspected cyanide intoxication (Sauer and Keim 2001).

Ancient origin of vitamin B_{12}

Because of its role in bacterial fermentation processes, Roth and coworkers (1996) concluded "that the original significance of B_{12}, and its remaining primary role in many modern bacteria, may be to support fermentation of small molecules" in order to "generate both an oxidizable compound and an electron sink for use in balancing

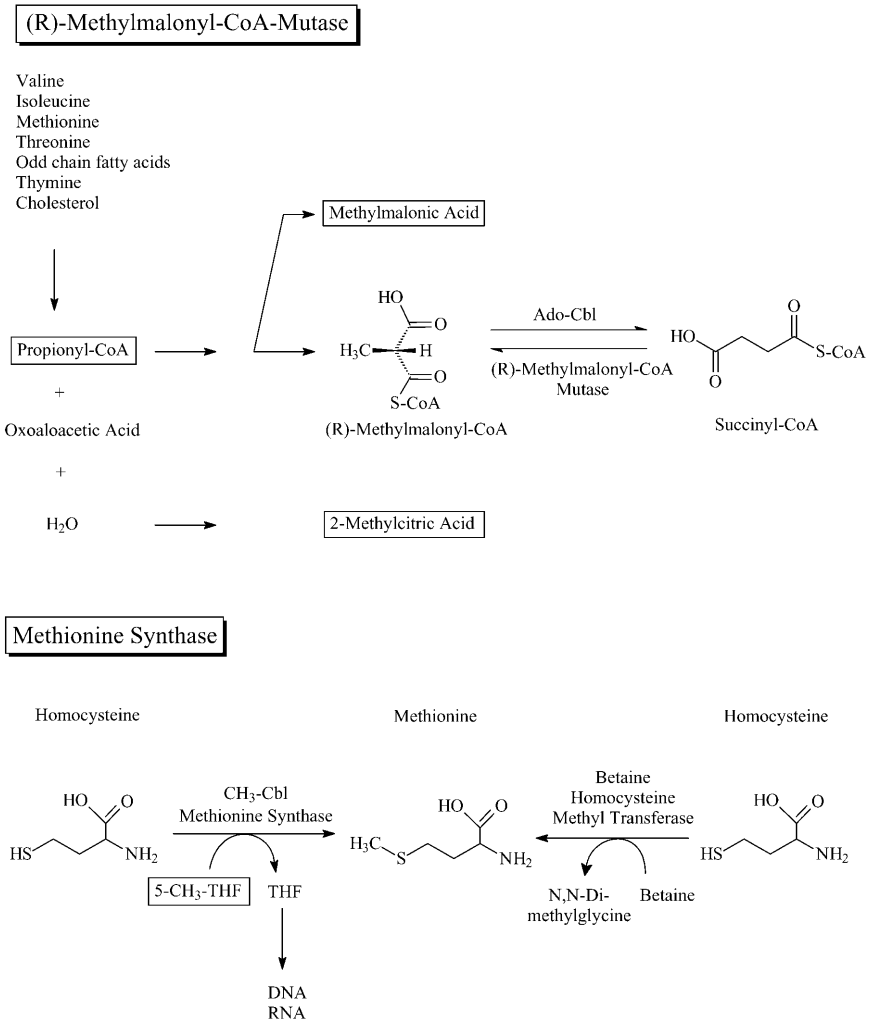

Fig. 3 Cobalamin-dependent reactions in humans and related metabolic pathways thought to be involved in vitamin B_{12}-related diseases. Some of these metabolites are accumulated during vitamin B_{12} deficiency. *CH_3-Cbl* Methylcobalamin, *5-CH_3-THF* 5-methyl-tetrahydrofolate, *THF* tetrahydrofolate

redox reactions." In an early emerging biotic environment, fermentation processes (and with them vitamin B_{12}) were of central importance for energy production. Other forms of anaerobic energy production, such as methanogenesis and various amino acid fermentations, all required vitamin B_{12}. It seems that vitamin B_{12}-function is linked to classic anaerobic metabolic processes. Even vitamin B_{12} biosynthesis provides evidence for an ancient function of the tetrapyrrole. In virtually all prokaryotes, except the α-group of proteobacteria, vitamin B_{12} biosynthesis starts with an aminoacyl-tRNA molecule (Jahn et al. 1992). Interestingly, it is also an RNA molecule that plays an intimate role in regulating expression of the genes for cobalamin biosynthesis in *Salmonella typhimurium* (Ravnum and Andersson 2001). Even adenosylcobalamin itself contains two ribonucleic acid handles. It has also been shown in so-called primitive earth experiments that porphyrinogens, such as those contributing to vitamin B_{12} formation, can be non-enzymatically synthesized from simple organic precursors (Hodgson and Ponnamperuma 1968). The initial biosynthetic routes from 5-aminolevulinic acid are identical for chlorophyll, heme, siroheme and vitamin B_{12}, until the formation of the first tetrapyrrole macrocycle, uroporphyrinogen III (Raux et al. 1999). Uroporphyrinogen III is asymmetric, which might be the fundamental feature for the ring contraction (carbon elimination) step occurring later in cobalamin biosynthesis (Eschenmoser 1988). This may reflect the possibility that the entire pathway was initially "invented" to synthesize vitamin B_{12} and the branches to synthesize chlorophylls and hemes were added later (Roth et al. 1996). Recently, evidence for an ancient heme biosynthetic pathway originating from the vitamin B_{12} branch of tetrapyrrole biosynthesis still existing in *Desulfovibrio vulgaris* was described (Ishida et al. 1998).

Taken together, these observations suggest that vitamin B_{12} emerged in a primitive RNA world (Benner et al. 1989), selected by its initial ability to support anaerobic fermentation of small molecules. Later, the development of siroheme allowed the use of simple inorganic ions as electron acceptors, documented by its role as a cofactor for sulfite and nitrite reductases. Finally, the establishment of chlorophyll and heme biosynthesis gave rise to the production and utilization of molecular oxygen (Roth et al. 1996). As the atmospheric levels of oxy-

gen increased, the metabolic dependency on vitamin B_{12} decreased. Secondary functions for vitamin B_{12} were developed, such as in methyl transfer or nucleotide reduction. Its original major role in anaerobic fermentation was restricted to ecological niches. Photosynthesis and oxygen respiration allowed the development of plants and animals, which do not form vitamin B_{12}. However, the unique chemistry mediated by vitamin B_{12} led to the secondary acquisition of vitamin B_{12}-dependent reactions by humans and animals.

The elucidation of the complete oxygen-dependent pathway to vitamin B_{12} in *P. denitrificans*

1993 marked the end of an era of at least 25 years of scientific research, when scientists were able to piece together the intricate pathway that leads to cobalamin biosynthesis. One of the major reasons for this breakthrough was due to the participation by three groups at RPR around Blanche, Crouzet and Vuilhorgne who, with major contributions from Battersby and Scott, were able to announce the elucidation of the complete biosynthetic pathway to vitamin B_{12} in the aerobic bacterium *P. denitrificans* (Blanche et al. 1995; Battersby 1998; Scott et al. 1999). When the studies on *P. denitrificans* started, *Propionibacterium shermanii* had been the reference organism for vitamin B_{12} biosynthesis research. The sequence of reactions leading from cobyrinic acid to cobalamin had been elucidated (Friedmann and Cagen 1970; Huennekens et al. 1982). Additionally, the pathway leading from 5-aminolaevulinic acid to precorrin-3 (now known as precorrin-3A) had been classified in detail. However, a huge gap remained between precorrin-3A and cobyrinic acid, representing a "black box", in which formation of nearly the whole corrin macrocycle was supposed to occur. This included essential steps of biosynthesis, like methylations, decarboxylation, cobalt insertion and ring contraction (Thibaut et al. 1998). Furthermore, neither a *cob* gene nor a *cob* mutant had ever been isolated from any micro-organism and none of the enzymes of the whole biosynthetic cobalamin-specific pathway had been purified to homogeneity (Blanche et al. 1995).

The key to the success of the French groups at RPR was mainly the choice of an aerobic micro-organism for their investigations in which cobalt insertion occurs late in biosynthesis. Thus, they were not forced to handle organic cobalto-complexes, which are most likely very unstable. In addition, the French scientists were the first to use the power of genetics and molecular biology, which in combination with enzymology, chemical synthesis, isotopic labeling and NMR spectroscopy, were the guarantors of success. To achieve this outstanding contribution to vitamin B_{12} biosynthesis research, 148 *Agrobacterium tumefaciens*, 24 *Pseudomonas putida* (Cameron et al. 1989) and more than 60 *P. denitrificans cob* mutants were generated (Blanche et al. 1998). A plasmid library representing more than 99% of the *P. denitrificans* genome was conjugated in *E. coli*, resulting in nearly 3,600 separate *E. coli* strains. In order to identify the *cob* genes of *P. denitrificans*, every one of the *A. tumefaciens* and *P. putida cob* mutants was tested by every *E. coli* strain for complementation via conjugation and investigated for cobalamin production. From such experiments, the researchers isolated about 78 kb of DNA and about half of this was sequenced, leading to the identification of 22 *cob* genes clustered in four complementation groups (Crouzet et al. 1990a, b, 1991; Cameron et al. 1991a, b). In order to draw the relationship between presumable *cob* genes and the biochemical functions of their encoded proteins enzyme activity assays, protein-expression schemes and chromatographic strategies for their recombinant purification to homogeneity were established. The N-terminal sequencing of at least 17 different Cob proteins was performed (Blanche et al. 1995, 1998). These studies were accompanied by the determination of intermediates in vitamin B_{12} biosynthesis accumulated in the various *cob* mutants via radioactivity- and fluorescence-detecting HPLC, leading to the structures of more than a dozen formerly unknown corrin and corrin precursor intermediates (Blanche et al. 1990, 1995; Debussche et al. 1990; Thibaut et al. 1998).

Vitamin B_{12} biosynthesis

As mentioned above, vitamin B_{12} biosynthesis is restricted to micro-organisms. Due to its complex chemical nature, more than 30 genes are required for the entire de novo biosynthesis of cobalamin, which amounts to about 1% of a typical bacterial genome (Roth et al. 1993). Two different biosynthetic routes for vitamin B_{12} exist in nature: (1) an aerobic, or more precisely an oxygen-dependent pathway that is found in organisms like *P. denitrificans* and (2) an anaerobic, oxygen-independent pathway investigated in organisms like *Bacillus megaterium*, *P. shermanii* and *Salmonella typhimurium*. Genes encoding enzymes contributing to the oxygen-dependent cobalamin biosynthesis are recognized by the prefix *cob*, while genes involved in the oxygen-independent pathway are usually named using the prefix *cbi*. A schematic outline of cobalamin biosynthesis and its oxygen-dependent versus oxygen-independent differences are shown in Fig. 4.

The biosynthesis of all tetrapyrrole derivatives in plants, archaea and the majority of bacteria, with the exception of the α-group of the proteobacteria, starts from the C-5 skeleton of glutamate. In the first step, tRNA-bound glutamate is reduced to glutamate-1-semialdehyde by glutamyl-tRNA reductase. The aldehyde is converted in a second step via an intramolecular shift of the amino group from the C-2 to the C-1 of glutamate-1-semialdehyde, to form 5-aminolevulinic acid, the first general precursor of all known tetrapyrroles. Two molecules of 5-aminolevulinic acid are condensed to generate the first pyrrole derivative, porphobilinogen. Four pyrrole molecules are polymerized, rearranged and then cyclized, to

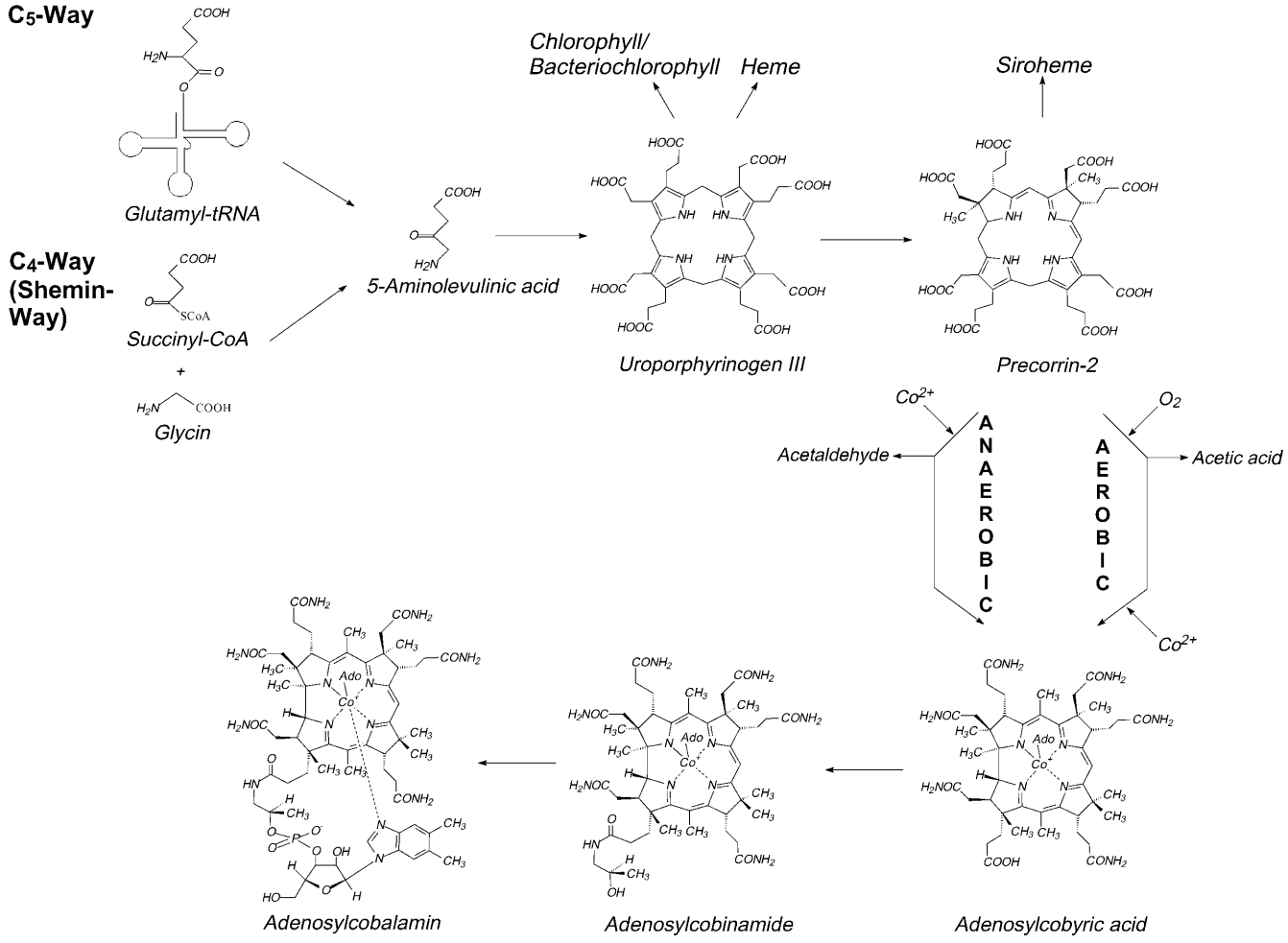

Fig. 4 Schematic representation of the aerobic and anaerobic cobalamin biosynthetic pathways

form uroporphyrinogen III, the first macrocyclic intermediate (summarized in Jahn et al. 1996).

Whilst decarboxylation of uroporphyrinogen III leads to the biosynthesis of hemes and chlorophylls, methylation of uroporphyrinogen III at C-2 and C-7 results in the synthesis of precorrin-2, a dimethylated dipyrrocorphin that is also the last common intermediate in the synthesis of coenzyme F430 and siroheme. The methyl groups added to the tetrapyrrole framework are derived from (s)-adenosyl-L-methionine; and the two methyl groups are added by the action of a single methyltransferase that is able to catalyze the addition to both positions (Goldman and Roth 1993; Woodcock et al. 1998). At precorrin-2, the two pathways for cobalamin biosynthesis diverge (Raux et al. 1999): in the aerobic pathway, precorrin-2 is methylated at C-20 by a further methyltransferase to give precorrin-3A while, in the anaerobic pathway, precorrin-2 is chelated with cobalt to give cobalt-precorrin-2, a reaction that is catalyzed in *S. enterica* by CbiK (Raux et al. 1997).

Thus, the oxygen-dependent and independent pathways for B_{12} synthesis are quite distinct: the oxygen-independent part of the pathway starts with the insertion of cobalt into precorrin-2, while this chelation reaction in the oxygen-dependent part occurs only after nine further reaction steps. Interestingly, the two cobalt-chelatases employed for these reactions are different, in that the oxygen-dependent pathway chelatase requires ATP, in contrast to its anaerobic counterpart which requires no high-energy equivalents.

Due to the early cobalt insertion of the oxygen-independent pathway, the majority of the intermediates are cobalto-complexes. Therefore, they require enzymes with different substrate specificities, compared with the metal-free intermediates of the oxygen-dependent pathway. A further difference between the two routes is the method employed to promote the ring-contraction process, with the removal of C-20 from the ring. Under aerobic conditions, the C-20 atom of precorrin-3A is oxidized by molecular oxygen, sustained by a Fe_4S_4 cluster-containing protein (CobG), with the subsequent release of C-20 as acetate. Under anaerobic conditions, the ring contraction process is likely to be mediated via the complexed cobalt ion with its ability to assume different valence states (+1 to +3) to assist in the oxidation, resulting in the release of C-20 as acetaldehyde. Indeed, Scott's group has identified a number of ring-contracted cobalt–corrinoid compounds, some of which are incorporated into cobyrinic acid (Scott et al. 1999).

While the B_{12} biosynthetic pathways diverge at precorrin-2, they do join again at the level of adenosyl-cobyric acid, which is converted into cobinamide by the attachment of an aminopropanol arm to the propionic acid side-chain of ring D. The lower nucleotide loop is attached by transferring the phosphoribosyl residue of nicotinic acid mononucleotide to DMBI. The resulting α-ribazole is finally covalently linked to GDP-activated adenosylcobinamide, thereby releasing GMP and giving rise to the completely manufactured coenzyme B_{12} molecule.

Vitamin B_{12} production

After 10 years of work, with more than 100 researchers, the full chemical synthesis of vitamin B_{12} was achieved by Woodward and Eschenmoser in 1973 (Eschenmoser 1974). This highly complicated synthesis, with about 70 synthesis steps, makes any industrial production of vitamin B_{12} by chemical methods far too technically challenging and expensive. Therefore, today vitamin B_{12} is exclusively produced by biosynthetic fermentation processes, using selected and genetically optimized micro-organisms.

Among the B_{12}-producing species are the following genera: *Aerobacter*, *Agrobacterium*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Clostridium*, *Corynebacterium*, *Flavobacterium*, *Micromonospora*, *Mycobacterium*, *Norcardia*, *Propionibacterium*, *Protaminobacter*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Streptomyces*, *Streptococcus* and *Xanthomonas* (Perlman 1959).

For the industrial production of cobalamin, it has been a common strategy to use random mutagenesis in order to generate strains that produce vitamin B_{12} in high yields. Generally this has been achieved by treating the appropriate micro-organisms with mutagenic agents like UV light, ethyleneimine, nitrosomethyluretane or *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine and selecting the strains with practical advantages, such as productivity, genetic stability, reasonable growth rates and resistance to high concentrations of toxic intermediates present in the medium. The most active producers of vitamin B_{12} are listed in Table 1 (Bykhovsky et al. 1998).

Because of their already naturally high vitamin B_{12} productivity and their rapid growth, mainly *Propionibacterium shermanii* and the inappropriately named *Pseudomonas denitrificans* strains are employed for industrial production. One would assume that the genus *Propionibacterium* should be preferred by industry, because, unlike *Pseudomonas denitrificans*, bacteria of this genus have obtained the GRAS (generally recognized as safe) status from the United States Food and Drug Administration.

Nevertheless, *P. denitrificans* is almost exclusively used in industrial processes by the main B_{12}-producing company, RPR (France), which fused with Hoechst AG (Germany) and is now known as Aventis. Combining random mutagenesis with methods of genetic engineering, a group of researchers at RPR (Blanche et al. 1995) created a highly effective vitamin B_{12}-producing strain. There is no official information on the productivity of this genetically engineered *P. denitrificans* strain. However, it is conceivable that its productivity may reach 300 mg/l. To a lesser extent, some directed genetic engineering of *Propionibacterium* strains has also been attempted, for instance via the overexpression of *cobA* (Powells et al. 1999).

All *Propionibacterium* strains employed for vitamin B_{12} production are microaerophilic and produce vitamin B_{12} in high yields only under very low oxygen concentrations. However, the biosynthesis of DMBI requires oxygen. Therefore, the bioprocess of vitamin B_{12} production using *Propionibacterium* strains is divided into two stages. In the first 3 days of fermentation, the bacteria are grown anaerobically to produce the vitamin B_{12} precursor cobamide, a vitamin B_{12} intermediate missing the DMBI moiety. Subsequently, vitamin B_{12} formation is completed by gentle aeration of the whole culture for 1–3 days, allowing the bacteria to undertake the oxygen-

Table 1 Species of microbial producers and microbiological processes recommended for producing vitamin B_{12} (Bykhovsky et al. 1998). The *Rhodopseudomonas protamicus* listed is a chimera obtained by fusion of protoplasts from *Protaminobacter ruber* and *R. spheroides*. Beside the *P. denitrificans* listed Rhône-Poulenc Rorer uses a genetically engineered strain which is supposed to reach a productivity of 100–300 mg/l

Species of micro-organism or microbiological process	Main component of culture medium	Conditions of fermentation	Vitamin B_{12} production (mg/l)
Propionibacterium freudenreichii	Glucose	Anaerobiosis, 5,6-dimethyl benzimidazole	206.0
Rhodopseudomonas protamicus	Glucose	5,6-dimethyl benzimidazole	135.0
Propionibacterium shermanii	Glucose	5,6-dimethyl benzimidazole	60.0
Pseudomonas denitrificans	Sucrose	Aerobiosis, betaine	60.0
Nocardia rugosa	Glucose	Aerobiosis	18.0
Rhizobium cobalaminogenum	Sucrose	Aerobiosis	16.5
Micromonospora sp.	Glucose	5,6-dimethyl benzimidazole	11.5
Streptomyces olivaceus	Glucose	5,6-dimethyl benzimidazole	6.0
Nocardia gardneri	Hexadecane	Aerobiosis	4.5
Butyribacterium methylotrophicum	Methanol	Anaerobiosis	3.6
Pseudomonas sp.	Methanol	5,6-dimethyl benzimidazole	3.2
Arthrobacter hyalinus	Isopropanol	5,6-dimethyl benzimidazole	1.1

dependent synthesis of the DMBI and to link it to cobamide. Furthermore, it is crucial to neutralize the accumulated propionic acid during the whole fermentation process, in order to maintain the production culture at pH 7, since the formation of propionic acid amounts to 10% of the fermentation volume (Eggersdorfer 1996).

In contrast to the *Propionibacterium* fermentation process, the production of vitamin B_{12} using *Pseudomonas denitrificans* parallels oxygen-dependent growth with high vitamin B_{12} production rates. The culture is aerated during the whole fermentation process of about 2–3 days at 30 °C and pH values are maintained at 6–7 (Eggersdorfer 1996; Scott 1998b).

Independent of the employed production strains and culture conditions, it seems to be necessary to add some essential compounds to the medium for efficient vitamin B_{12} biosynthesis. The addition of cobalt ions and DMBI are frequently described. Sometimes, further additions of potential precursors like glycine, threonine, δ-aminolevulinic acid or compatible solutes like betaine (found in high contents in sugar beet molasses) and choline prove to be beneficial.

Usually, the whole broth or an aqueous suspension of harvested cells is heated at 80–120 °C for 10–30 min at pH 6.5–8.5 in order to extract the vitamin B_{12}. The conversion to cyanocobalamin is obtained by treating the heated broth or cell suspension with cyanide or thiocyanate (Spalla et al. 1989). After clarification of the whole solution, via e.g. filtration or treatment with zinc hydroxide, vitamin B_{12} is precipitated by the addition of auxiliaries like tannic acid or cresol. This procedure leads to a product of about 80% purity, which is used as animal feed additive. Further purification via different extraction steps, using organic solvents like cresol, carbon tetrachloride and water/butanol, is often supplemented by adsorption to ion exchangers or activated carbon. Finally, vitamin B_{12} is crystallized by the addition of organic solvents, leading to a product of recommended quality for food and pharmaceutical applications (Eggersdorfer 1996).

Genetic engineering of *P. denitrificans*

The detailed elucidation of vitamin B_{12} biosynthesis in *P. denitrificans* allowed the researchers of RPR a production strain improvement, which was not restricted to simple random mutagenesis. However, even RPR could not omit to exploit the power of this genetic process. After 10 years of multiple rounds of random mutagenesis, the vitamin B_{12} production of a single *P. denitrificans* strain was increased approximately 100-fold (Blanche et al. 1998). Probably based on this initial vitamin B_{12} production strain, RPR used its profound knowledge of vitamin B_{12} biosynthesis to systematically construct several genetically engineered *P. denitrificans* production strains. The very detailed European patent 0516647 B1 (Blanche et al. 1998) describes the amplification of the eight genes in the *cobF–cobM* operon in *P. denitrificans* and of the *cobA* and *cobE* genes. The term “amplification” is used to describe an increase in gene copy number by the use of multicopy plasmids. In *P. denitrificans*, a 30% increase in cobalamin production was detected, caused by amplification of the *cobF–cobM* gene cluster. An additional productivity enhancement of 20% was achieved by increasing the *cobA* and *cobE* copy number. The manipulation of transcriptional and translational control elements in order to increase the amount of expressed Cob proteins, like strong inducible promoters, highly efficient ribosomal binding sites and terminator sequences was also outlined by RPR (Blanche et al. 1997, 1998).

To overcome the substrate inhibition of the *cobA*-encoded methyltransferase, which catalyzes the first dedicated steps of vitamin B_{12} biosynthesis, RPR suggests the heterologous expression of the *corA* gene from *Methanobacterium ivanovii*, encoding an enzyme devoid of substrate inhibition (Blanche et al. 1998). The utilization of heterologous genes for improved vitamin B_{12} production was extended to the *Rhodobacter capsulatus* genes, named *bluB, bluE* and *bluF*. Normally DMBI, the lower ligand of the cobalt central ion, is one limiting factor in vitamin B_{12} biosynthesis. It is usually added to fermentation media, as mentioned above. Cellular DMBI biosynthesis was significantly enhanced by *trans*-expression of the *bluB* gene of *R. capsulatus* (Blanche et al. 1997). Stimulation of vitamin B_{12} production by (R)-1-amino-2-propanol and O-phospho-L-threonine, a new intermediate assumed to take the place of (R)-1-amino-2-propanol in the formation of the nucleotide loop of vitamin B_{12}, was also detected. The positive effects of O-phospho-L-threonine on vitamin B_{12} production was also achieved by *trans*-expression of the *bluE* and *bluF* genes of *R. capsulatus* (Blanche et al. 1997).

The combination of undirected mutagenesis and directed genetic manipulation allowed RPR to establish an efficient production process to cover more than 80% of the world production of vitamin B_{12}.

References

Abeles RH, Lee HA Jr (1961) An intramolecular oxidation-reduction requiring a cobamide coenzyme. J Biol Chem 236: 2347–2350

Allen LH, Casterline J (1994) Vitamin B_{12} deficiency in elderly individuals: diagnosis and requirements. Am J Clin Nutr 60: 12–14

Barker HA, Weissbach H, Smyth RD (1958) A coenzyme containing pseudovitamin B_{12}. Proc Natl Acad Sci USA 44:1093–1097

Battersby AR (1994) How nature builds the pigments of life: the conquest of vitamin B_{12}. Science 264:1551–1557

Battersby AR (1998) B_{12}-Biosynthesis in an aerobic organism: how the pathway was elucidated. In: Kräutler B, Arigoni D, Golding BT (eds) Vitamin B_{12} and B_{12}-proteins. Wiley/VCH, Weinheim, pp 47–61

Becher B, Müller V, Gottschalk G (1992) N^5-Methyl-tetrahydromethanopterin: coenzyme M methyltransferase of *Methanosarcina* strain Gü1 is an Na^+-translocating membrane protein. J Bacteriol 1774:7656–7660

Benner SA, Ellington AD, Thauer A (1989) Modern metabolisms as a palimpset of the RNA world. Proc Natl Acad Sci USA 86:7054–7058

Blakely R (1965) Cobamides and ribonucleotide reduction. I. cobamide stimulation of ribonucleotide reduction in extracts of *Lactobacillus leichmanii*. J Biol Chem 240:2173–2180

Blakely R, Barker H (1964) Cobamide stimulation of the reduction of ribotides to deoxyribotides in *Lactobacillus leichmanii*. Biochem Biophys Res Commun 16:391–397

Blanche F, Thibaut D, Couder M, Muller JC (1990) Identification and quantitation of corrinoid precursors of cobalamin from *Pseudomonas denitrificans* by high-performance liquid chromatography. Anal Biochem 189:24–29

Blanche F, Cameron B, Crouzet J, Debussche L, Thibaut D, Vuilhorgne M, Leeper FJ, Battersby AR (1995) Vitamin B_{12}: wie das Problem seiner Biosynthese gelöst wurde. Angew Chem 107:421–452

Blanche F, Cameron B, Crouzet J, Debussche L, Thibaut D (1997) Rhône-Poulenc Rorer. World Patent 97/43421

Blanche F, Cameron B, Crouzet J, Debussche L, Levy-Schil S, Thibaut D (1998) Rhône-Poulenc Biochimie. Eur Patent 0516647 B1

Blaut M, Müller V, Gottschalk G (1992) Energetics of methanogenesis studied in vesicular systems. J Bioenerg Biomembr 24:529–546

Bradbeer C (1965) The clostridial fermentations of choline and ethanolamine. I. Preparation and properties of cell-free extracts. J Biol Chem 240:4669–4674

Bronstrup A, Hages M, Prinz-Langenohl R, Pietrzik K (1998) Effects of folic acid and combinations of folic acid and vitamin B_{12} on plasma homocysteine concentrations in healthy, young women. Am J Clin Nutr 68:1104–1110

Burke S, Krzycki J (1995) Involvement of the "A" isozyme of methyltransferase II and the 29 kilodalton corrinoid protein in methanogenesis from monomethylamine. J Bacteriol 177:4410–4416

Bykhovsky VY, Zaitseva NI, Eliseev AA (1998) Tetrapyrroles: diversity, biosynthesis, and biotechnology. Appl Biochem Microbiol 34:1–18

Cameron B, Briggs K, Pridmore S, Brefort G, Crouzet J (1989) Cloning and analysis of genes involved in coenzyme B_{12} biosynthesis in *Pseudomonas denitrificans*. J Bacteriol 171:547–557

Cameron B, Guilhot C, Blanche F, Cauchois L, Rouyez MC, Rigault S, Levy-Schil S, Crouzet J (1991a) Genetic and sequence analysis of a *Pseudomonas denitrificans* DNA fragment containing two *cob* genes. J Bacteriol 173:6058–6065

Cameron B, Blanche F, Rouyez MC, Bisch D, Famechon A, Couder M, Cauchois L, Thibaut D, Debussche L, Crouzet J (1991b) Genetic analysis, nucleotide sequence, and products of two *Pseudomonas denitrificans cob* genes encoding nicotinate-nucleotide: dimethylbenzimidazole phosphoribosyltransferase and cobalamin (5′-phosphate) synthase. J Bacteriol 173:6066–6073

Crouzet J, Cauchois L, Blanche F, Debussche L, Thibaut D, Rouyez MC, Rigault S, Mayaux JF, Cameron B (1990a) Nucleotide sequence of a *Pseudomonas denitrificans* 5.4-kilobase DNA fragment containing five *cob* genes and identification of structural genes encoding S-adenosyl-L-methionine: uroporphyrinogen III methyltransferase and cobyrinic acid a,c-diamide synthase. J Bacteriol 172:5968–5979

Crouzet J, Cameron B, Cauchois L, Rigault S, Rouyez MC, Blanche F, Thibaut D, Debussche L (1990b) Genetic and sequence analysis of an 8.7-kilobase *Pseudomonas denitrificans* fragment carrying eight genes involved in transformation of precorrin-2 to cobyrinic acid. J Bacteriol 172:5980–5990

Crouzet J, Levy-Schil S, Cameron B, Cauchois L, Rigault S, Rouyez MC, Blanche F, Debussche L, Thibaut D (1991) Nucleotide sequence and genetic analysis of a 13.1-kilobase-pair *Pseudomonas denitrificans* DNA fragment containing five cob genes and identification of structural genes encoding cob(I)alamin adenosyltransferase, cobyric acid synthase, and bifunctional cobinamide kinase-cobinamide phosphate guanylyltransferase. J Bacteriol 173:6074–6087

Debussche L, Thibaut D, Cameron B, Crouzet J, Blanche F (1990) Purification and characterisation of cobyrinic acid a,c-diamide synthase from *Pseudomonas denitrificans*. J Bacteriol 172: 6239–6244

Delva MD (1997) Vitamin B_{12} replacement. To B_{12} or not to B_{12}? Can Fam Physician 43:917–922

Dixon M, Huang S, Matthews RG, Ludwig ML (1996) The structure of the C-terminal domain of methionine synthase: presenting S-adenosylmethionine for reductive methylation of B_{12}. Structure 4:1263–1275

Drennan C, Huang S, Drummond JT, Matthews RG, Ludwig ML (1994) How a protein binds B_{12}: a 3.0A X-ray structure of B_{12}-binding domains of methionine synthase. Science 266: 1669–1674

Duda J, Pedziwilk Z, Zodrow K (1967) Studies on the vitamin B_{12} content of the leguminous plants. Acta Microbiol Pol 6:233–238

Eggersdorfer M (1996) Vitamins. In: Elvers B, Hawkinds S (eds) Ullmann's encyclopedia of industrial chemistry, vol 27A, 5th edn. VCH, Weinheim, pp 443–613

Eschenmoser A (1974) Organische Naturstoffsynthese heute, Vitamin B_{12} als Beispiel. Naturwissenschaften 61:513–525

Eschenmoser A (1988) Vitamin B_{12}: experiments concerning the origin of its molecular structure. Angew Chem Int Ed Engl 27: 5–39

Ferry J (1992) Methane From acetate. J Bacteriol 174:5489–5495

Ferry J (ed) (1993) Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York

Ferry J (1995) CO dehydrogenase. Annu Rev Microbiol 49:305–333

Finke RG (1998) Coenzyme B_{12}-based chemical precedent for Co-C bond homolysis and other key elementary steps. In: Kräutler B, Arigoni D, Golding BT (eds) Vitamin B_{12} and B_{12}-proteins. Wiley/VCH, Weinheim, pp 383–402

Forage RG, Foster MA (1982) Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B_{12}-dependent glycerol and diol dehydratases. J Bacteriol 149:413–419

Frey PA (2001) Radical mechanisms of enzymatic catalysis. Annu Rev Biochem 70:121–148

Friedmann HC, Cagen LM (1970) Microbial biosynthesis of B_{12}-like compounds. Annu Rev Microbiol 24:159–208

Goldman BS, Roth JR (1993) Genetic structure and regulation of the *cysG* gene in *Salmonella typhimurium*. J Bacteriol 175: 1457–1466

Guest JR, Friedmann S, Woods DD, Smith EL (1962) A methyl analogue of cobamide coenzyme in relation to methionine synthesis by bacteria. Nature 195:340–342

Harms LL, Thauer RK (1998) EPR Spectroscopic evidence that, in the energy conserving methyltransferase complex from methanogenic archaea, a histidine residue is ligated to the cobamide-cobalt. In: Kräutler B, Arigoni D, Golding BT (eds) Vitamin B_{12} and B_{12}-proteins. Wiley/VCH, Weinheim, pp 157–165

Hodgkin DC, Pickworth J, Robertson JH, Trueblood KN, Prosen RJ, White J (1955) Structure of vitamin B_{12}. Nature 176: 325–328

Hodgkin DC, Kamper J, Mackay M, Pickworth M, Trueblood KN, White JG (1956) Structure of vitamin B_{12}. Nature 178:64–70

Hodgkin DC, Kamper J, Lindsey J, McKay M, Pickworth J, Robertson JH, Brink Shoemaker C (1957) The structure of vitamin B_{12}. I. An outline of the crystallographic investigation of vitamin B_{12}. Proc R Soc Lond Ser A 242:228–263

Hodgson GW, Ponnamperuma C (1968) Prebiotic porphyrin genesis: porphyrins from electric discharge in methane, ammonia and water vapor. Proc Natl Acad Sci USA 59:22–28

Huennekens FM, Vitols KS, Fujii K, Jacobsen DW (1982) Biosynthesis of cobalamin coenzymes. In: Dolphin D (ed) B_{12}, vol 1. Wiley, New York, pp 145–167

Ishida T, Yu L, Akutsu H, Ozawa K, Kawanishi S, Seto A, Inubushi T, Sano S (1998) A primitive pathway of porphyrin biosynthesis and enzymology in *Desulfovibrio vulgaris*. Proc Natl Acad Sci USA 9:4853–4858

Jahn D, Verkamp E, Söll D (1992) Glutamyl-transfer RNA: a precursor of heme and chlorophyll biosynthesis. Trends Biochem Sci 6:215–218

Jahn D, Hungerer C, Troup B (1996) Ungewöhnliche Wege und umweltregulierte Gene der bakteriellen Hämbiosynthese. Naturwissenschaften 83:389–400

Joosten E, Berg A van den, Riezler R, Naurath HJ, Lindenbaum J, Stabler SP, Allen RH (1993) Metabolic evidence that deficiencies of vitamin B_{12} (cobalamin), folate, and vitamin B_6 occur commonly in elderly people. Am J Clin Nutr 58:468–476

Jordan A, Reichard P (1998) Ribonucleotide reductases. Annu Rev Biochem 67:71–98

Keltjens J, Vogels G (1993) Conversion of methanol and methylamines to methane and carbon dioxide. In: Ferry J (ed) Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York, pp 253–302

Lawrence JG, Roth JR (1995) Evolution of coenzyme B_{12} synthesis among enteric bacteria: evidence for loss and reacquisition of a multigene complex. Genetics 142:11–24

Lenhert PG, Hodgkin DC (1961) Structure of the 5,6-dimethylbenzimidazolylcobamide coenzyme. Nature 192:937–938

Lindenbaum J, Rosenberg IH, Wilson PW, Stabler SP, Allen RH (1994) Prevalence of cobalamin deficiency in the Framingham elderly population. Am J Clin Nutr 60:2–11

Ljungdahl L, Wood H (1982) Acetate biosynthesis. In: Dolphin D (ed) B_{12}, vol 2. Wiley, New York, pp 165–202

Mancia F, Keep NJ, Nakagawa A, Leadlay PF, McSweeney S, Rasmussen B, Bösecke P, Diat P, Evans PR (1996) How coenzyme B_{12} radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. Structure 4:339–350

McCaddon A, Kelly CL (1994) Familial Alzheimer's disease and vitamin B_{12} deficiency. Age Ageing 23:334–337

Müller V, Blaut M, Gottschalk G (1993) Bioenergetics of methanogenesis. In: Ferry J (ed) Methanogenesis: ecology, physiology, biochemistry and genetics. Chapman & Hall, New York, pp 360–406

Obradors N, Badia J, Baldoma L, Aguilar J (1988) Anaerobic metabolism of the L-rhamnose fermentation product 1,2-propanediol in *Salmonella typhimurium*. J Bacteriol 170:2159–2162

Perlman D (1959) Microbial synthesis of cobamides. Adv Appl Microbiol 1:87–122

Peston JM (1977) Leucine 2,3 aminomutase: a cobalamin-dependent enzyme present in bean seedlings. Science 195:301–302

Poirot C, Kengen S, Valk E, Keltjens J, Drift C van der, Vogels G (1987) Formation of methylcoenzyme M from formaldehyde by cell free extracts of *Methanobacterium thermoautotrophicum*. Evidence for the involvement of a corrinoid-containing methyltransferase. FEMS Microbiol Lett 40:7–13

Pouwels PH, Van Luijk N, Jore JPM, Luiten RGM (1999) Gist-Brocades. World Patent 99/67356

Ragsdale S (1991) Enzymology of the acetyl-CoA pathway of CO_2 fixation. Crit Rev Biochem Mol Biol 26:261–300

Raux E, Thermes C, Heathcote P, Rambach A, Warren MJ (1997) A role for *Salmonella typhimurium* cbiK in cobalamin (vitamin B_{12}) and siroheme biosynthesis. J Bacteriol 179:3202–3212

Raux E, Schubert HL, Roper JM, Wilson KS, Warren MJ (1999) Vitamin B_{12}: insights into biosynthesis's mount improbable. Bioorg Chem 27:100–118

Ravnum S, Andersson DI (2001) An adenosyl-cobalamin (coenzyme B_{12})-repressed translational enhancer in the *cob* mRNA of *Salmonella typhimurium*. Mol Microbiol 39:1585–1594

Reichard P (1993) From RNA to DNA, why so many reductases? Science 260:1773–1777

Reitzer R, Gruber K, Jogl G, Wagner UG, Bothe H, Buckel W, Kratky C (1999) Glutamate mutase From *Clostridium cochlearium*: the structure of a coenzyme B_{12}-dependent enzyme provides new mechanistic insights. Struct Fold Des 8:891–902

Rickes EL, Brink NG, Koniuszy FR, Wood TR, Folkers K (1948a) Crystalline vitamin B_{12}. Science 107:396–397

Rickes EL, Brink NG, Koniuszy FR, Wood TR, Folkers K (1948b) Comparative data on vitamin B_{12} from liver and from a new source. Science 108:634–635

Roth JR, Lawrence JG, Rubenfield M, Dieffer-Higgins S, Church GM (1993) Characterization of the cobalamin (vitamin B_{12}) biosynthetic genes of *Salmonella typhimurium*. J Bacteriol 175:3303–3316

Roth JR, Lawrence JG, Bobik TA (1996) Cobalamin (coenzyme B_{12}): synthesis and biological significance. Annu Rev Microbiol 50:137–181

Sauer SW, Keim ME (2001) Hydroxocobalamin: improved public health readiness for cyanide disasters. Ann Emerg Med 37: 635–41

Scarlett FA, Turner MJ (1976) Microbial metabolism of amino alcohols. Ethanolamine catabolism mediated by coenzyme B_{12}-dependent ethanolamine ammonia-lyase in *Escherichia coli* and *Klebsiella aerogenes*. J Gen Microbiol 95:173–176

Scott AI (1998a) How nature synthesizes B_{12} without oxygen. Discoveries along the ancient, anaerobic pathway. In: Kräutler B, Arigoni D, Golding BT (eds) Vitamin B_{12} and B_{12}-proteins. Wiley/VCH, Weinheim, pp 81–100

Scott AI, Roessner CA, Santander PJ (1999) B_{12} Biosynthesis: the anaerobic pathway. In: Banerjee R (ed) Chemistry and biochemistry of B_{12}. Wiley, New York, pp 537–556

Scott JW (1998b) Vitamin B_{12}. In: Kroschwitz JI, Howe-Grant M (eds) Kirk-Othman encyclopedia of chemical technology, vol 25, 4th edn. Wiley, New York, pp 193–217

Shibata N, Masuda J, Tobimatsu T, Toraya T, Suto K, Morimoto Y, Yasuoka N (1999) A new mode of B_{12} binding and the direct participation of a potassium ion in enzyme catalysis: X-ray structure of diol dehydratase. Struct Fold Des 7:997–1008

Spalla C, Grein A, Garofano L, Ferni G (1989) Microbial production of vitamin B_{12}. In: Vandamme E J (ed) Biotechnology of vitamins, pigments and growth factors. Elseviewer, London pp 257–284

Stabler SP (1999) B_{12} and nutrition In: Banerjee R (ed) Chemistry and biochemistry of B_{12}. Wiley, New York, pp 343–365

Stabler SP, Lindenbaum J, Savage DG, Allen RH (1993) Elevation of serum cystathionine levels in patients with cobalamin and folate deficiency. Blood 81:3404–3413

Stroinsky A, Schneider Z (1987) Cobamide dependent enzymes. In: Schneider Z, Stroinsky A (eds) Comprehensive B_{12}. de Gruyter, Berlin, pp 225–266

Stupperich E (1993) Recent advances in elucidation of biological corrinoid functions. FEMS Microbiol Rev 12:349–356

Stupperich E, Eisinger HJ, Albracht SPJ (1990) Evidence for a super-reduced cobamide as the major corrinoid fraction in vivo and a histidine residue as a cobalt ligand of the p-cresolyl cobamide in the acetogenic bacterium *Sporomusa ovata*. Eur J Biochem 193:105–109

Thibaut D, Blanche F, Cameron B, Crouzet J, Debussche L, Remy E, Vuilhorgne M (1998) Vitamin B_{12} biosynthesis in *Pseudomonas denitrificans*. In: Kräutler B, Arigoni D, Golding BT (eds) Vitamin B_{12} and B_{12}-proteins. Wiley/VCH, Weinheim, pp 63–79

Toraya T, Honda S, Fukui S (1979) Fermentation of 1,2-propanediol and 1,2-ethanediol by some genera of Enterobacteriaceae, involving coenzyme B_{12}-dependent diol dehydratase. J Bacteriol 139:39–47

Van Rooyen JP, Mienie LJ, Erasmus E, De Wet WJ, Ketting D, Duran M, Wadman SK (1994) Identification of the stereoisomeric configurations of methylcitric acid produced by Si-citrate synthase and methylcitrate synthase using capillary gas chromatography-mass spectrometry. J Inherit Metab Dis 17:738–747

Watanabe F, Nakano Y, Tamura Y, Yamanaka H (1991) Vitamin B_{12} metabolism in a photosynthesizing green alga, *Chlamydomonas reinhardtii*. Biochim Biophys Acta 1075:36–41

Watanabe F, Tamura Y, Stupperich E, Nakano Y (1993) Uptake of cobalamin by *Euglena* mitochondria. J Biochem (Tokyo) 114:793–799

Weiss DS, Thauer RK (1993) Methanogenesis and the unity of biochemistry. Cell 6:819–822

Wood H, Ragsdale W, Pezacka E (1986) The acetyl-CoA pathway: a newly discovered pathway of autotrophic growth. Trends Biochem Sci 11:14–17

Woodcock SC, Raux E, Levillayer F, Thermes C, Rambach A, Warren MJ (1998) Effect of mutations in the transmethylase and dehydrogenase/chelatase domains of sirohaem synthase (CysG) on sirohaem and cobalamin biosynthesis. Biochem J 330:121–129

Zelder O, Beatrix B, Kroll F, Buckel W (1995) Coordination of a histidine residue of the protein-component S to the cobalt atom in coenzyme B_{12}-dependent glutamate mutase from *Clostridium cochlearium*. FEBS Lett 369:252–254