Metabolic engineering of cobalamin (vitamin B$_{12}$) production in *Bacillus megaterium*

Rebekka Biedendieck,1 Marco Malten,2 Heiko Barg,3 Boyke Bunk,4 Jan-Henning Martens,5 Evelyne Deery,1 Helen Leech,1 Martin J. Warren1 and Dieter Jahn4*

1Protein Science Group, Department of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK.
2Novozymes A/S, Krogshøjvej 36, 2880 Bagsvaerd, Denmark.
3BASF SE, Carl-Bosch-Strasse 38, 67056 Ludwigshafen, Germany.
4Institute of Microbiology, Technische Universität Braunschweig, Spielmannstrasse 7, 38106 Braunschweig, Germany.
5ALEXIS Corporation, Industriestrasse 17, 4415 Lausen, Switzerland.

**Summary**

Cobalamin (vitamin B$_{12}$) production in *Bacillus megaterium* has served as a model system for the systematic evaluation of single and multiple directed molecular and genetic optimization strategies. Plasmid and genome-based overexpression of genes involved in vitamin B12 biosynthesis, including *cbiX*, *sirA*, modified *hemA*, the operons *hemAXCDBL* and *cbiXJCDETLFGAcysGAcbiYbtuR*, and the regulatory gene *fnr*, significantly increased cobalamin production. To reduce flux along the heme branch of the tetrapyrrole pathway, an antisense RNA strategy involving silencing of the *hemZ* gene encoding coproporphyrinogen III oxidase was successfully employed. Feedback inhibition of the initial enzyme of the tetrapyrrole biosynthesis, HemA, by heme was overcome by stabilized enzyme overproduction. Similarly, the removal of the B$_{12}$ riboswitch upstream of the *cbiXJCDETLFGAcysGAcbiYbtuR* operon and the recombinant production of three different vitamin B$_{12}$ binding proteins (glutamate mutase GlmS, ribonucleotide triphosphate reductase RtpR and methionine synthase MetH) partly abolished B$_{12}$-dependent feedback inhibition. All these strategies increased cobalamin production in *B. megaterium*. Finally, combinations of these strategies enhanced the overall intracellular vitamin B$_{12}$ concentrations but also reduced the volumetric cellular amounts by placing the organism under metabolic stress.

**Introduction**

In order to improve the production of commercially important metabolites, a number of different metabolic engineering strategies can be employed. These include the overexpression, deletion, modification and up- and down-regulation of genes associated directly or indirectly with the process. Strategies for the manipulation of gene expression comprise the utilization of strong inducible promoters, the removal of repressory promoter elements, the improvement of positive translation signals and the removal of inhibitory structures, such as riboswitches (Rygus and Hillen, 1991; Shrader et al., 1993; Ravnum and Andersson, 2001; Yakandawala et al., 2008). These approaches can lead to the overproduction of enzymes associated with the metabolic control of the biosynthesis of the compound of interest. Furthermore, to direct precursor molecules common to several metabolic processes along a specific pathway, repression mechanisms can be employed to downregulate the competing processes (Tatarko and Romeo, 2001; Shen and Liao, 2008). To provide additional reducing equivalents (NADH/NADPH) cells can be engineered and grown on suitable carbon sources to ensure an increase in the reduced cofactor ratio (Bäumchen et al., 2007). Protein engineering approaches allow for the modification of substrate specificity, the removal of feedback inhibitory sites and the change of other central catalytic properties of enzymes (Kang et al., 2007; Beine et al., 2008). Here, we have applied a systematic evaluation of different metabolic engineering strategies to the production of cobalamin (vitamin B$_{12}$) in *Bacillus megaterium*.

Cobalamin is an important nutrient. The naturally occurring cobalamins, namely 5′-deoxyadenosylcobalamin (coenzyme B$_{12}$) and methylcobalamin, are essential cofactors for the human enzymes methylmalonyl CoA mutase (Mancia et al., 1996) and methionine synthase (Drennan et al., 1994), respectively. Vitamin B$_{12}$ is unique among the vitamins as it appears to be solely produced by prokaryotes (Roth et al., 1996; Croft et al., 2005). The structural complexity of the molecule is reflected in an equally complex total chemical synthesis, which requires
more than 60 steps (Woodward, 1973; Eschenmoser and Wintner, 1977). For this reason, commercial production of vitamin B₁₂ is achieved by bacterial fermentation processes mainly using genetically improved strains of Propionibacterium shermanii and Pseudomonas denitrificans (Martens et al., 2002). In nature, cobalamins are synthesized via a branch of the modified tetrapyrrole pathway, involving around 30 enzyme-mediated steps, encoded by genes that account for up to 1% of a bacterial genome (Roth et al., 1993).

Two major routes exist for the biosynthesis of the co-enzyme form of cobalamin, which are referred to as the oxygen-dependent and oxygen-independent pathways. The majority of the oxygen-dependent biosynthetic pathway from P. denitrificans was elucidated by researchers at Rhône Poulenc (today Sanofi-Aventis) in order to develop their production strain (Thibaut et al., 1998). An alternative oxygen-independent route is found in bacteria, such as Salmonella enterica (Jeter and Roth, 1987) and Propionibacterium freudenreichii (Blanche et al., 1993). The two pathways are distinct mainly in the synthesis of the corrin-ring component of cobalamin, where they diverge at the dimethylated derivative of uroporphyrinogen III and merge at the formation of adenosylcobamic acid. The genetic nomenclature for cobalamin biosynthesis is also somewhat confusing. The genes for the aerobic pathway are given the prefix cob, whereas the genes for the anaerobic pathway, up to the biosynthesis of cobinamide, are prefixed cbi and the genes involved in the transformation of cobinamide into adenosylcobalamin are prefixed cob. Reviews on cobalamin biosynthesis and its biotechnological production are available for more detailed perusal (Martens et al., 2002; Escalante-Semerena and Warren, 2008).

The Gram-positive bacterium B. megaterium was one of the first vitamin B₁₂ producers employed biotechnologically (Wolf and Brey, 1986; Vary, 1992; Vary et al., 2007). Molecular genetic approaches helped in the localization of the genes required for cobalamin biosynthesis (Brey et al., 1986; Wolf and Brey, 1986; Vary, 1994; Raux et al., 1998a,b; Martens et al., 2002). Since then, major parts of this biosynthetic pathway in B. megaterium have been genetically and biochemically characterized (Brey et al., 1986; Wolf and Brey, 1986; Vary, 1994; Raux et al., 1998a,b; Martens et al., 2002). Even though B. megaterium has been classified as a strict aerobe, the organism employs the oxygen-independent pathway for cobalamin synthesis (Raux et al., 1998a). During the past decade, B. megaterium has also been used for the high yield production of recombinant proteins (Burger et al., 2003; Barg et al., 2005; Malten et al., 2005; 2006; Yang et al., 2006; Biedendieck et al., 2007a,b). This has been made possible by the discovery and development of multiple autonomously replicating and chromosomal integratable plasmids, which permit for the intra- and extracellular production of proteins of interest. One of the strong, inducible promoter systems employed in this approach is based on the regulatory elements of the operon encoding xylose-utilization enzymes (Ryagus and Hillen, 1991; Ryagus et al., 1991; Biedendieck et al., 2007a), whereby the Pₓyl₄ promoter, controlled by the XylR repressor protein, allows for xylose-inducible gene expression.

Here, we combine the natural ability of B. megaterium to biosynthesize cobalamin with the recently developed genetic tools to test, systematically, a range of strategies for cobalamin production.

Results and discussion

Enhancing vitamin B₁₂ precursor formation by biosynthetic enzyme engineering and overproduction

B. megaterium makes two major tetrapyrrole derivatives, heme and cobalamin, and they share a common pathway from glutamyl-tRNA to uroporphyrinogen III. In B. megaterium, this section of the pathway is encoded by the hemAXCDBL operon (Raux et al., 2003). The expression of these genes is known to be strictly regulated at the transcriptional and posttranslational levels in organisms such as Escherichia coli, S. typhimurium and Pseudomonas aeruginosa (Darie and Gunsalus, 1994; Moser et al., 2002; Schobert and Jahn, 2002). The first enzyme of the pathway, glutamyl-tRNA reductase (HemA), is a major control point of tetrapyrrole biosynthesis. Besides feedback inhibition at the transcriptional level of hemA by iron-protoporphyrin IX (protopheme), the protein itself is subject to protopheme-dependent proteolytic degradation as a second mode of metabolic control (Schobert and Jahn, 2002). To try and increase flux along the pathway, a strategy of overproducing a stable version of HemA was employed. This was achieved by engineering a version of HemA with two lysine residues at positions +3 and +4. Such changes have previously been observed to lead to increased protein stability in S. typhimurium (Wang et al., 1999) (Fig. 1). Thus, the B. megaterium hemAKK gene was cloned downstream of the xylose-inducible promoter Pₓyl₄ in the integrative plasmid pHBihemAkk, which, due to its temperature-sensitive origin of replication, was replicated independently of the chromosome at non-permissive temperatures below 37°C.

When transformed B. megaterium and upon induction with xylose, the presence of the plasmid resulted in the production of HemAkk, which was produced at levels detectable by SDS-PAGE analysis (data not shown). The effect of this increased recombinant HemAkk production resulted in a 13.5-fold (10.8-fold) rise in the intracellular vitamin B₁₂ concentration to 0.54 μg l⁻¹ OD₅78nm⁻¹.
(2.81 μg l⁻¹) (Table 1) in comparison with the wild-type strain (0.04 μg l⁻¹ OD₅₇₈nm⁻¹ and 0.26 μg l⁻¹, respectively).

The hemA KK was subsequently integrated into the chromosomal hemA locus by a single cross-over event by raising the growth temperature of the strain carrying pHBihemAKK to 42°C resulting in the construction of strain HBBm1 (Fig. 2A). As a consequence, the expression of the complete chromosomal B. megaterium hemA KKXCDBL operon was brought under the control of the strong inducible promoter PₓylA. When xylose was added to HBBm1, it led to a change in the cellular soluble proteome when compared with that of wild-type DSM509 as observed by PAGE analysis (Fig. 2B). Moreover, 5 h after induction the cells of strain HBBm1 developed a red-brown pigmentation as opposed to the normal yellowish colour observed for the parental wild-type strain DSM509 (Fig. 2C), consistent with the accumulation of porphyrin. This genetic manipulation resulted in an increase of vitamin B12 to 1.59 μg l⁻¹ OD₅₇₈nm⁻¹ and to 8.51 μg l⁻¹ for the HBBm1 strain, an increase of 39.8- and 32.7-fold, respectively, in comparison with the wild-type strain.

During prolonged cultivation at 37°C, HBBm1 was found to be genetically unstable, as the xylose-inducible promoter upstream of the hemAKKXCDBL operon was eliminated. This problem was overcome by growing the strain at 42°C.

Fig. 1. Alignment of the N-terminal amino acid sequence of glutamyl-tRNA reductase HemA from S. typhimurium and B. megaterium.

The amino acid sequences of the N-terminus of HemA from S. typhimurium and B. megaterium are given in the first two lines. In the third line, the two lysines (K) for which novel coding sequence was introduced into the hemA gene sequence of B. megaterium via site-directed mutagenesis are shown in grey shading. Conserved amino acids are given in black shading.

Fig. 2. Molecular strategy for the overexpression of the chromosomal hemA KKXCDBL operon.

A. After construction of the integrative plasmid pHBihemA KK (II), the plasmid was integrated into the B. megaterium chromosome (I) via a single cross-over recombination (III). Plasmid elements of pHBihemA KK are the xylose-inducible promoter PₓylA, the temperature-sensitive origin of plasmid replication oriF, the gene essential for plasmid replication repF and the gene responsible for erythromycin resistance eryr.

Plasmid elements for plasmid replication in E. coli are the origin of replication colE1 and the β-lactamase gene amp' responsible for the ampicillin resistance.

B. B. megaterium DSM509 (line 1) and the new mutant strain HBBm1 (line 2) were cultivated at the presence of 0.23% (w/v) xylose in LB medium at 42°C. After induction of recombinant gene expression with 0.5% (w/v) xylose, samples were taken and prepared for SDS-PAGE gel analysis as described in Experimental procedures. Overproduced proteins are marked at relevant size. M = Marker (Dalton Mark VII).

C. Cells of B. megaterium DSM509 (left-hand site, yellow cells) and HBBm1 (DSM509 with integrated pHBihemA KK) (right-hand site, red cells) were harvested 5 h after induction of recombinant overexpression of the hemA KKXCDBL operon. The strong formation of tetrapyrroles is indicated by the red colour.

Fig. 2.
Table 1. Summary of intracellular vitamin B₁₂ amounts for the various optimization experiments with *B. megaterium*.

| *B. megaterium* strain | Relevant genotype | Strategy Description | Vitamin B₁₂ (mg ml⁻¹ OD⁻¹ 1578nm) | Factor | Vitamin B₁₂ (µg l⁻¹) | Factor |
|------------------------|------------------|-----------------------|-----------------------------------|--------|-----------------------|--------|
| DSM509                 | Wild-type        | –                     | 0.04                              | 1.0    | 0.26                  | 1.0    |
| DSM509                 | Wild-type        | Cobalt addition       | 0.16                              | 4.0    | 0.56                  | 2.2    |
| DSM509                 | Wild-type        | Anaerobic growth      | 0.20                              | 5.0    | 0.08                  | 0.3    |
| DSM509-pHBlHemAₘ        | Pₓyl hemAₘ (plasmid) | Overexpression of feedback deregulated HemA | 0.54 | 13.5 | 2.81 | 10.8 |
| HBBm1                  | Pₓyl hemAₘXCDBL (genome) | Overexpression of chromosomal hemAₘXCDBL operon and feedback deregulation of HemA | 1.59 | 39.8 | 8.51 | 32.7 |
| DSM509-pWH1520sirA      | Pₓyl sir (plasmid) | Overexpression of sirA | 0.18 | 4.5 | 1.10 | 4.2 |
| DSM509-pHBbcBX         | Pₓyl cbiX (plasmid) | Overexpression of cbiX | 0.35 | 8.8 | 0.60 | 2.3 |
| DSM509-pHBbcBX (cobalt addition) | Pₓyl cbiX (plasmid) | Overexpression of cbiX (cobalt addition) | 0.82 | 20.5 | 1.59 | 6.1 |
| HBBm3                  | Pₓyl cbiXJCDETLF | Overexpression of chromosomal cbiX operon | 0.47 | 11.8 | 0.85 | 3.5 |
| HBBm3 (cobalt addition) | Pₓyl cbiXJCDETLF | Overexpression of chromosomal cbiX operon (cobalt addition) | 0.79 | 19.8 | 1.24 | 4.8 |
| DSM509-pWH1520ashemZ    | Pₓyl ashemZ (plasmid) | Downregulation of heme via antisense hemZ RNA | 0.05 | 1.2 | 0.31 | 1.2 |
| DSM509-pWH1520fnr       | Pₓyl fnr (plasmid) | Overexpression of fnr | 0.19 | 4.8 | 0.97 | 3.7 |
| HBBm1-pWH1520ashemZ (3 h/5 h after induction) | Pₓyl hemAₘXCDBL (genome) | Overexpression of chromosomal hemAₘXCDBL operon, feedback deregulation of HemA and downregulation of heme via antisense hemZ RNA | 1.32/0.92 | 33.022.9 | 4.50/3.53 | 17.3/13.6 |
| HBBm1-pWH1520sirA (3 h/5 h after induction) | Pₓyl hemAₘXCDBL (genome) | Overexpression of chromosomal hemAₘXCDBL operon, feedback deregulation of HemA and overexpression of sirA | 1.17/1.49 | 29.1/37.2 | 3.52/4.93 | 13.5/19.0 |
| DSM509-pMM1522metH⁺     | Pₓyl metH⁺ (plasmid) | Overexpression of metH⁺ and feedback deregulation | 0.06 | 1.5 | 0.41 | 1.6 |
| DSM509-pMM1522rtpR      | Pₓyl rtpR (plasmid) | Overexpression of rtpR and feedback deregulation | 0.19 | 4.7 | 1.14 | 4.4 |
| DSM509-pMM1522glmS      | Pₓyl glmS (plasmid) | Overexpression of glmS and feedback deregulation | 0.20 | 4.9 | 1.20 | 4.7 |

Employed *B. megaterium* strains, their relevant genotypes, the tested up- and downregulation strategies (marked with ↑ or ↓), intracellular vitamin B₁₂ concentrations per cell expressed as µg l⁻¹ OD⁻¹ 1578nm, the volumetric amount of B₁₂ as µg l⁻¹, as well as the incremental factors related to vitamin B₁₂ concentrations achieved with the wild-type strain DSM509 are given. Cultivation was in 50 ml cultures in LB medium. If indicated, 250 µM cobalt chloride were added. Recombinant gene expression was induced with 0.5% (w/v) xylose. All vitamin B₁₂ measurements were done in triplicates with error margins of 10%.
Reduction of the uroporphyrinogen III flux towards heme via antisense RNA inhibition of hemZ expression

In order to reduce flux towards heme along the branched pathway and also to reduce the possibility of harmful high hemZ concentrations (Nakahigashi et al., 1991), attempts were made to engineer this branch of the pathway. Interestingly, it is the enzyme coproporphyrinogen III oxidase that appears to be a regulated step (Homuth et al., 1999; Schobert and Jahn, 2002). This explains why feeding E. coli or P. aeruginosa cultures with 5-aminolevulinic acid, the tetrapyrrole precursor that is taken up by bacteria, often leads to coproporphyrin excretion (Doss and Philipp-Dormston, 1971; Doss, 1974).

Two classes of oxygen-independent oxidases, HemN and HemZ, are utilized for the protoporphyrinogen IX formation in B. subtilis (Homuth et al., 1999). By using a PCR approach with degenerate primers, the B. megaterium genome was probed for the presence of the corresponding genes (hemN and hemZ), but only hemZ was detected. More recently, the finished genome sequence of B. megaterium revealed the presence of both, a hemZ 1506 bp and a hemN 1152 bp. In order to direct more of the metabolic flux of the common precursor uroporphyrinogen III along the cobalamin pathway, the flow through the heme biosynthetic pathway needed to be downregulated (Fig. 3). For this, an antisense RNA (asRNA) strategy for the transient inactivation of hemZ was chosen. To accomplish this,

Fig. 3. Optimization strategies for biosynthesis of vitamin B\(_{12}\) in B. megaterium. The biosynthesis of vitamin B\(_{12}\) and heme with the relevant steps to our approach is shown. Green arrows show the flow of intermediates to vitamin B\(_{12}\) while red arrows indicate the competing pathways for heme and siroheme formation. Steps in vitamin B\(_{12}\) biosynthesis, which were recombinantly manipulated via the control of the corresponding genes by the xylose-inducible promoter, are indicated by ‘P\(_{\text{xyH}}\)’, followed by the corresponding gene of interest. (+) indicates an increase of cobalamin levels due to the overexpression of the recombinant gene while (−) indicates a reduced flux towards the heme due to the overexpression of an antisense RNA. Red arrows crossed by green bars show released feedback inhibition. Genes/proteins with the prefix cob/Cob are involved in the cobalamin biosynthesis of B. megaterium. Genes/proteins with the prefix hem/Hem are involved in tetrapyrrole biosynthesis. hemAKK/HemAKK – mutated form of the B. megaterium hemA gene/HemA protein; metH*/MetH* – gene of the vitamin B\(_{12}\) binding domain of MetH/vitamin B\(_{12}\) binding domain of MetH from E. coli; rtpR/RtpR – gene of the ribonucleotide triphosphate reductase/ribonucleotide triphosphate reductase from L. delbrueckii; glmS/GlmS – gene of the small subunit of the glutamate mutase/small subunit of the glutamate mutase of C. cochlearium.
a 129 bp long DNA sequence coding for an *antisense* hemZ RNA (*ashemZ*) was designed and placed under the control of *P* <sub>xylA</sub>, creating plasmid pWH1520ashemZ. The asRNA included 82 nt upstream of the start codon of hemZ as well as the first 47 nt of hemZmRNA. The *ashemZ* : hemZ mRNA duplex renders the ribosome binding site inaccessible for the ribosome (Zadra et al., 2000) and thereby prevents hemZmRNA translation.

To evaluate the physiological consequences of *ashemZ* expression, we determined the coproporphyrinogen III : protoporphyrinogen IX as well as the heme and vitamin B<sub>12</sub> concentration in a strain harbouring pWH1520ashemZ and compared it with an appropriate wild-type control strain. For simplicity, coproporphyrinogen III and protoporphyrinogen IX were oxidized by hydrogen peroxide treatment to their corresponding porphyrins, which are more easily detectable via fluorescence spectroscopy. Coproporphyrin III shows two distinct emission peaks at 579 and 620 nm when excited by light of wavelength 409 nm while protoporphyrin IX is characterized by a single peak at 633 nm (Fig. 4A). Figure 4B shows a fluorescence spectrum of cell free extracts of *B. megaterium* strain DSM509 carrying pWH1520ashemZ in comparison with a spectrum obtained from *B. megaterium* DSM509 carrying the empty vector pWH1520. A comparison of the peak maxima reveals that more coproporphyrinogen III is accumulated in the strain producing *ashemZ* (Fig. 4C). Furthermore, the accumulation of coproporphyrinogen III results in decreased heme formation in the *ashemZ*-producing *B. megaterium* (data not shown). Higher levels of coproporphyrinogen III are also likely to inhibit the HemE reaction by product inhibition (Jones and Jordan, 1993). Significantly, though, this antisense approach led to an increase in the intracellular vitamin B<sub>12</sub> concentration by 20% (0.05 μg l<sup>-1</sup> OD<sub>578nm</sub><sup>-1</sup> and 0.31 μg l<sup>-1</sup>, respectively) (Table 1).

The repression of heme biosynthesis by an asRNA is the first successful example of use of this strategy in *B. megaterium* and is one of only a few reported antisense strategies in bacteria. This externally controlled switch-on and -off of chromosomal gene expression offers an alternative method to gene knockout approaches, especially for essential genes where some basal level of transcription and translation is required for cell viability. In the case of *B. megaterium* heme biosynthesis, the asRNA approach was necessary as the bacterium requires heme for growth but does not efficiently take up heme from the environment.

**Directing uroporphyrinogen III flux towards vitamin B<sub>12</sub> biosynthesis by recombinant overexpression of sirA**

After enhancing the intracellular concentration of the common tetrapyrrolic primogenitor, uroporphyrinogen III, by modulation of the hemAXCDBL operon, attempts to modulate flux towards vitamin B<sub>12</sub> were made (Fig. 3). Uroporphyrinogen III is directed towards cobalamin synthesis and away from the porphyrin branch by transformation into precorrin-2, a bis-methylated derivative (Raux et al., 2000; Martens et al., 2002). This reaction is catalyzed by a SAM-dependent uroporphyrinogen III methyltransferase that mediates the two transmethylation reactions at C2 and C7 of the tetrapyrrole template. The gene encoding this enzyme from *B. megaterium* was first cloned and published as cobA (Robin et al., 1991). Later, it was renamed *sirA* after isolation of the *sirABC* operon for siroheme biosynthesis (Leech et al., 2002). To direct uroporphyrinogen III flux towards vitamin B<sub>12</sub> biosynthesis, the *sirA* gene was overexpressed from an autonomously replicating plasmid under the control of *P* <sub>xylA</sub> (pWH1520sirA). This approach resulted in a 4.5- and 4.2-fold increase (0.18 μg l<sup>-1</sup> OD<sub>578nm</sub><sup>-1</sup> and 1.1 μg l<sup>-1</sup>) in the intracellular vitamin B<sub>12</sub> concentration in the *B. megaterium* strain DSM509 carrying pWH1520sirA (Table 1).

**Influence of cobalt addition, cobaltochelatase CbiX overproduction, and cobI operon overexpression on vitamin B<sub>12</sub> biosynthesis**

Cobalt is the central ion of all cobalamin. It is a trace element and its concentration in the growth medium may represent a limiting factor for vitamin B<sub>12</sub> production. Hence, wild-type DSM509 was grown in complex medium supplemented with 250 μM cobalt chloride. This addition resulted in a 4- (0.16 μg l<sup>-1</sup> OD<sub>578nm</sub><sup>-1</sup>) and 2.2-fold (0.56 μg l<sup>-1</sup>) increase, respectively (Table 1).

If the level of cobalt is limiting for cobalamin production, then the enzyme necessary for cobalt integration might also represent a further bottleneck in the biosynthesis. In the anaerobic pathway cobalt is inserted into the intermediate sirohydrochlorin. By overproduction of SirA, an increase of the fluorescence could be recognized, which is associated with an increase of precorrin-2 and sirohydrochlorin levels. In an analogous situation to uroporphyrinogen III, the latter compound can be directed to one of two different end-products, either siroheme or vitamin B<sub>12</sub> (Fig. 3). Siroheme is a minor tetrapyrrole required solely for assimilating sulfate and nitrite reductanes (Leech et al., 2002). A ferrochelatase, SirB, inserts iron into siroheme, whereas the cobaltochelatase, CbiX, inserts cobalt into the same molecule assigning it to cobalamin biosynthesis (Raux et al., 2003). The CbiX gene is the third gene of the 10.5 kb *cobI* operon necessary for vitamin B<sub>12</sub> production in *B. megaterium*. This operon encodes *cbiI*, *cbiH*, *cbiH<sub>co</sub>*, *cbiX*, *cbiJ*, *cbiC*, *cbiD*, *cbiET*, *cbiL*, *cbiF*, *cbiG*, *cbiA*, *cysG<sup>3</sup>* and *cbiY* necessary for cobyrinic acid a,c-diamide formation (Raux et al., 1998a). Furthermore, in *S. enterica*, the end-
product, adenosylcobalamin, binds to a riboswitch on the mRNA just upstream of the main cobi operon. Via this riboswitch, the translation of the cobi mRNA is inhibited (Ravnum and Andersson, 2001). Analysis of the upstream region of the cobi operon in B. megaterium (http://sanger.ac.uk) also revealed the presence of a possible riboswitch motif, localized between 573 bp to 95 bp upstream to the translational start of the first gene (cbiW) in this operon.

The cbix gene was cloned into the chromosomally integratable plasmid pHBinT to construct pHBicbiX. This autonomously replicating plasmid mediated xylose-
inducible overexpression of cbiX (P<sub>xyl</sub>-cbiX) after it was transferred into <i>B. megaterium</i> strain DSM509, which resulted in significantly increased intracellular CbiX levels (data not shown). In contrast to wild-type <i>B. megaterium</i>, this strain also showed a rise in the final cell density when the growth medium was supplemented with cobalt chloride. The intracellular cobalamin concentrations in <i>B. megaterium</i> DSM509 transformed with pHBicbiX were 8.8× (0.35 μg l<sup>−1</sup> OD<sub>578nm</sub>) and 20.5-fold (0.82 μg l<sup>−1</sup> OD<sub>578nm</sub>) higher compared with the wild-type DSM509 when the bacterium was grown without and with the supplementation of cobalt, respectively (Table 1). The volumetric B<sub>12</sub> concentrations were enhanced 2.3- (0.6 μg l<sup>−1</sup>) and 6.1-fold (1.59 μg l<sup>−1</sup>), respectively.

Next, we aimed to over-express the majority of the cobI operon in parallel with the elimination of the feedback inhibition mediated by the riboswitch. For this purpose, the xylose-inducible promoter P<sub>xyl</sub> localized on pHBicbiX was inserted into the chromosomal cobI operon upstream of the cbiX gene leading to strain HBBm3. The first two genes of the operon, cbiW and cbiH<sub>60</sub>, remained under the control of the natural promoter of the operon, but the subsequent genes now come under the control of P<sub>xyl</sub>. After xylose induction to enhance the expression of the cobI operon starting at the cbiX gene, no differences in the intracellular protein pattern of wild-type strain compared with HBBm3 were observed (data not shown). However, an 11.8- (3.5-) and 19.8-(4.8)-fold increase of cellular vitamin B<sub>12</sub> concentrations [0.47 μg l<sup>−1</sup> OD<sub>578nm</sub>] (0.85 μg l<sup>−1</sup>) without and 0.79 μg l<sup>−1</sup> OD<sub>578nm</sub> (1.24 μg l<sup>−1</sup>) with cobalt addition, respectively) were observed. The increase in cobalamin production is similar to the recombinant strain DSM509 carrying pHBicbiX. However, the copy number of the cbiX gene is much higher via plasmid-mediated expression compared with the chromosomal expression of the cbiX containing cobI operon. We conclude from these results that cobalt chelation (catalyzed by CbiX) likely represent a major limiting step in the cobalamin biosynthetic pathway. Moreover, chromosomal overexpression of the whole operon also leads to an increase of intracellular cobalamin concentration, indicating the importance of this part of the pathway for metabolic control.

**Induction of cobalamin production by overproduction of the oxygen global regulator Fnr**

As <i>B. megaterium</i> houses an oxygen-independent cobalamin biosynthetic pathway (Raux <i>et al.</i>, 1998a), it was hypothesized that an anaerobic environment may enhance the cobalamin production process as the pathway generates many oxygen-sensitive intermediates. To support this idea it was observed that <i>B. megaterium</i> had a 5-fold induction of cobalamin production (0.2 μg l<sup>−1</sup> OD<sub>578nm</sub>) when grown anaerobically. Similar observations have also been made with cobalamin formation in <i>S. enterica</i> (Andersson and Roth, 1989). However, the anaerobic conditions also resulted in a 16-fold reduction in the cell mass, thereby decreasing the volumetric vitamin B<sub>12</sub> concentration to 0.08 μg l<sup>−1</sup> compared with 0.26 μg l<sup>−1</sup> for the aerobic cultivation conditions (Table 1).

In <i>B. subtilis</i>, the model organism for Gram-positive bacilli, the oxygen global regulator Fnr mediates anaerobic gene expression (Marino <i>et al.</i>, 2001; Reents <i>et al.</i>, 2006a,b). In order to combine aerobic growth yields with the efficiency of anaerobic vitamin B<sub>12</sub> production, <i>B. subtilis</i> fnr was recombinantly overexpressed in aerobically grown <i>B. megaterium</i> DSM509 by cloning the gene under the control of P<sub>xyl</sub>. <i>B. megaterium</i> transformed with pWH1520fnr showed a 4.8-fold increase in the amount of cobalamin per cell (0.19 μg l<sup>−1</sup> OD<sub>578nm</sub>) and significantly a 3.7-fold increase in the volumetric cobalamin amount (0.97 μg l<sup>−1</sup>) (Table 1). Aerobically produced <i>B. subtilis</i> Fnr revealed the same effect as anaerobic growth conditions on cobalamin biosynthesis. Hence, cobalamin production seems to be either directly or indirectly influenced by the anaerobic regulator Fnr.

The recently completed sequencing of the <i>B. megaterium</i> genome offered the opportunity to screen for potential Fnr binding sites using the Virtual Footprint tool and the PRODORIC database (Münch <i>et al.</i>, 2005). Fnr binding sites were found upstream of the operons encoding the NADH-dependent nitrite reductase NasD, the lactate transporter YeaB, the lactate dehydrogenase Ldh and the quinol oxidase QoxA. For <i>B. subtilis</i> it was shown that anaerobic lactate fermentation and nitrite reduction significantly contribute to the overall growth due to their NAD regeneration electron sink function (Hoffmann <i>et al.</i>, 1998). Both activities are also integral parts of the aerobic nitrogen assimilation and carbon overflow metabolism (Cruz Ramos <i>et al.</i>, 2000). In <i>B. subtilis</i>, the quinol oxidase operon qoxABCD encodes the major oxygen-dependent terminal oxidase cytochrome ca<sub>3</sub> (Winstedt and von Wachenfeld, 2000). In <i>B. megaterium</i>, Fnr-dependent additional induction of this system under aerobic conditions might also contribute to the overall ATP production, growth yield and consequently vitamin B<sub>12</sub> biosynthesis.

**Overproduction of vitamin B<sub>12</sub> binding proteins to overcome feedback inhibition**

We decided to investigate if overproduction of a cobalamin binding protein could be used to overcome the effect of end-product inhibition, for example, as mediated by the riboswitch described earlier. To this end, three different proteins containing known vitamin B<sub>12</sub> binding domains were selected. These included the vitamin B<sub>12</sub> binding
domain of *E. coli* methionine synthase MetH (further named MetH*,  \( M = 28 \, 000 \)), which was shown to bind vitamin B\(_{12}\) with a \( K_d \) of 2.8 \( \mu M \), (Luschinsky et al., 1992; Hall et al., 2001), the small subunit of glutamate mutase (GlmS) from the Gram-positive organism *Clostridium cochlearium* (Zelder et al., 1994), which has a \( M \) of 14 800 and a \( K_d \) (GlmS/vitamin B\(_{12}\)) of 5.6 \( \pm 0.7 \) mM (Eichmüller et al., 2001), and ribonucleotide triphosphate reductase (RtpR), which utilizes an alternative mechanism of vitamin B\(_{12}\) binding with a \( K_d \) (RtpR/vitamin B\(_{12}\)) of 1.3 \( \mu M \) (Booker and Stubbe, 1993; Chen et al., 2003). In the case of the latter, the RtpR from the Gram-positive *Lactobacillus delbrueckii* was chosen.

Genes for all three cobalamin binding proteins were cloned individually under \( P_{xyl} \) control into pMM1522 and tested for recombinant overexpression in *B. megaterium* DSM509. Recombinant overexpression of \( \text{metH}^* \) resulted in a 1.5-fold (1.6-fold) increase in the intracellular cobalamin level to 0.06 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \) (0.41 \( \mu g \, l^{-1} \)) while over-expression of \( \text{rtpR} \) and \( \text{glmS} \) led to a 4.7- and 4.9-fold (4.4- and 4.7-fold) enhancement of intracellular vitamin B\(_{12}\) concentration of 0.19 and 0.2 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \) (1.14 and 1.2 \( \mu g \, l^{-1} \)), respectively. This increase of the intracellular vitamin B\(_{12}\) concentration was slightly lower than that achieved by the deletion of the riboswitch.

The overproduction of cobalamin binding proteins was not as successful as other strategies. One reason for this may come from the relatively weak interaction of the cofactor with the proteins, especially in the case of GlmS, which has a \( K_d \) of 5.6 \( \pm 0.7 \) mM (Eichmüller et al., 2001). Furthermore, no strong overproduction of any recombinant protein was observed when the cell protein profile was analyzed by SDS-PAGE (data not shown). The poor protein production is likely due to the codon bias of the corresponding gene, especially as codon bias is of significant importance for recombinant gene expression in *B. megaterium* (Bäumchen et al., 2007; Yang et al., 2007). Although determination of the codon adaptation index of these genes with the JCat program (Grote et al., 2005) revealed a reasonable value of 0.5 for the \( \text{glmS} \) gene, the values of 0.2 and 0.22 obtained for \( \text{rtpR} \) and \( \text{metH}^* \) suggest that codon optimization of these genes would lead to considerable improvement in gene expression. Nevertheless, using cobalamin binding proteins to soak up excess vitamin B\(_{12}\) from the system represents a successful strategy for enhanced product formation.

**Combinations of various optimization strategies**

To enhance further intracellular cobalamin production, combinations of overproduction strategies were tested. Hence, the strong chromosomal expression of the \( \text{hemA}_{aoXCDBL} \) operon in *B. megaterium* HBBm1 was combined with the autonomously replicating plasmid pWH1520ashemZ for the repression of competing heme biosynthesis. However, growth studies with the resulting *B. megaterium* mutant strain HBBm1 carrying pWH1520ashemZ reached only 69% of the final cell density of HBBm1. Nonetheless, a 2.9-fold increase in vitamin B\(_{12}\) production rate (0.66 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \, h^{-1} \)) was found within the first 2 h compared with that of *B. megaterium* HBBm1 without the ashemZ plasmid (0.23 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \, h^{-1} \)). Two hours after induction of expression of the recombinant gene, a cobalamin concentration of 1.32 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \) (4.5 \( \mu g \, l^{-1} \)) was measured. However, 5 h after induction, the concentration decreased again to 0.92 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \) (3.53 \( \mu g \, l^{-1} \)). It would seem that the antisense strategy coupled with increased flux along the B\(_{12}\) branch is detrimental to heme synthesis. The decrease in cobalamin concentration after 5 h may be caused by antisense RNA instability or feedback inhibition of B\(_{12}\).

Next, the expression of the \( \text{hemA}_{aoXCDBL} \) operon and the sirA gene, both placed under the control of the xylose-inducible promoter \( P_{xyl} \), were combined to enhance the biosynthetic steps leading to sirohydrochlorin (Fig. 3). For this purpose, HBBm1 carrying \( P_{xyl} \) upstream of the chromosomal \( \text{hemA}_{aoXCDBL} \) operon was transformed with the autonomously replicating plasmid pWH1520sirA carrying sirA under the control of the same promoter. Again, this *B. megaterium* plasmid carrying strain revealed reduced growth reaching only 57% of the final cell density of HBBm1. As with HBBm1 transformed with pWH1520ashemZ, a three times higher cobalamin production level was achieved during the first 2 h after induction of recombinant gene expression by HBBm1 overproducing SirA compared with HBBm1 without the SirA plasmid (0.66 and 0.23 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \, h^{-1} \), respectively) with a cobalamin concentration of 1.17 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \) (3.52 \( \mu g \, l^{-1} \)) (Table 1). Nevertheless, the maximal cobalamin concentrations of 1.49 and 1.59 \( \mu g \, l^{-1} \, OD_{578\,nm}^{-1} \), respectively, achieved after 5 h are in the same range with both strains, while the volumetric amount was less due to reduced growth (4.93 vs. 8.51 \( \mu g \, l^{-1} \)). The initial enhanced cobalamin production is clear proof of the principle for the approach of increase of sirA expression in HBBm1. Feedback inhibition of the subsequent biosynthetic pathway steps by the accumulation of the final product may be responsible for values observed after 5 h.

The plasmid-encoded production of recombinant ashemZ RNA and of recombinant SirA with the high-level synthesis of antibiotic resistance mediating proteins as well as the induced expression of the \( \text{hemA}_{aoXCDBL} \) operon appear to constitute an increased metabolic burden for the host cell (Glick, 1995). The resulting low cell density reduced the volumetric amount of vitamin B\(_{12}\) in *B. megaterium* strain HBBm1 carrying
Table 2. Strains used in this study.

| Name          | Description                                                                 | Reference or source                  |
|---------------|-----------------------------------------------------------------------------|--------------------------------------|
| E. coli DH10B | F: mcrA Δ(mrr-hsdRMS-mcrBC) e880lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK Δ(rpsL). nupG | Gibco Life Technologies               |
| B. megaterium | Wild-type strain, vitamin B₁₂ producer                                      |                                      |
| DSM509        | Mutant of DSM509, chromosomal overexpression of the hemA₆xXCDBL operon under xylose controlled PₓylA | DSMZ*, Braunschweig, Germany          |
| HBBm1         | Mutant of DSM509, chromosomal overexpression of the cbiX gene and the downstream located genes of the cobl operon under xylose controlled PₓylA | This work                            |
| HBBm3         | Mutant of DSM509, chromosomal overexpression of the cbiX gene and the downstream located genes of the cobl operon under xylose controlled PₓylA | This work                            |
| B. subtilis   | Wild-type strain, vitamin B₁₂ producer                                      |                                      |
| 168 L. delbrueckii ssp. lactis | Wild-type strain, vitamin B₁₂ producer                                          |                                      |
| DSM20076      | Wild-type strain, vitamin B₁₂ producer                                      |                                      |
|               | Wild-type strain, vitamin B₁₂ producer                                      |                                      |

*DSMZ, German Collection of Microorganisms and Cell Cultures.

pWH1520ashemZ as well as in HBBm1 transformed with pWH1520sirA compared with strain HBBm1 (3.53 and 4.93 μg l⁻¹, respectively, versus 8.51 μg l⁻¹).

Conclusion

Cobalamin biosynthesis in B. megaterium has served as a challenging model for the systematic engineering of a biochemical pathway using biosynthetic genes, enhanced enzyme stabilization, competing pathway downregulation and the elimination of end-product inhibition. All strategies were successful, resulting in a maximal 39.8-fold increase in the intracellular cobalamin concentration. Combining these individual strategies further increased the production level, but was limited due to the high metabolic burden placed upon the organism.

Experimental procedures

DNA manipulation for plasmid construction

Molecular biological methods were outlined previously (Sambrook and Russell, 2001). The B. megaterium strains, constructed plasmids and oligonucleotides used for cloning are listed in Tables 2–4.

The hemA gene was isolated from genomic B. megaterium DNA using primers hemA⁻₅ for and hemA⁺rev and ligated

Table 3. Plasmids used in this study.

| Name          | Description                                                                 | Reference or source                  |
|---------------|-----------------------------------------------------------------------------|--------------------------------------|
| pWH1520       | Shuttle vector for cloning in E. coli (Ap') and recombinant gene expression under xylose control | Rygus and Hillen (1991)               |
| pMM1522       | Shuttle vector for cloning in E. coli (Ap') and recombinant gene expression under xylose control | Malten et al. (2006)                 |
| pWH1967K      | Cloning, expression and integration vector for Bacillus spp.; Ap', Tc', Nm', ori²  | Schmiedel et al. (1997)               |
| pHBintE       | Cloning, expression and integration vector for Bacillus spp. with xylose-inducible promoter PₓylA Ap', Tc', ori² | Barg et al. (2006)                   |
| pHBintT       | Cloning, expression and integration vector for Bacillus spp. with xylose-inducible promoter PₓylA Ap', Tc', ori² | This work                            |
| pHBintN       | Cloning, expression and integration vector for Bacillus spp. with xylose-inducible promoter PₓylA Ap', Nm', ori² | This work                            |
| pOZ3          | Vector with 800 bp fragment of C. cochlearium glmS (gene coding for the small subunit of the glutamate mutase) in pJF118HE | Zelder et al. (1994)                 |
| pHBihemA₆x    | pHBintE-derivative: vector for chromosomal integration of the xylose-inducible promoter PₓylA downstream of the B. megaterium hemA₆xXCDBL operon | Barg et al. (2005)                   |
| pWH1520ashemZ | pWH1520-derivative: vector for the recombinant production of antisense RNA against 136 bp of the 5’-mRNA of B. megaterium hemZ including the ribosome binding site | This work                            |
| pWH1520sirA   | pWH1520-derivative: vector for recombinant overexpression of B. megaterium sirA in B. megaterium | This work                            |
| pHBcbiX       | pHBintN-derivative: vector for chromosomal integration of the xylose-inducible promoter PₓylA downstream of the cbiX gene in the cobi operon of B. megaterium | This work                            |
| pWH1520frn    | pWH1520-derivative: vector for recombinant overexpression of B. subtilis fnr in B. megaterium | This work                            |
| pMM1522meH⁺   | pMM1522-derivative: vector for recombinant overexpression of E. coli meH⁺ in B. megaterium | This work                            |
| pMM1522glmS   | pMM1522-derivative: vector for recombinant overexpression of C. cochlearium glmS in B. megaterium | This work                            |
| pMM1522trpR   | pMM1522-derivative: vector for recombinant overexpression of L. delbrueckii ssp. lactis rtpR in B. megaterium | This work                            |
Table 4. Oligos used in this study.

| Name       | Sequence 5′-3′ |
|------------|---------------|
| intN_for   | ggtatccatggtcgcaacctagagaagttggcgg |
| intN_rev   | ccaaaagcgtttgatatcaccagagaaggggag |
| intT_for   | aagactgtacacaagctgctgcagagtagtgg |
| intT_rev   | aaaaaagctttgagactcagacagaaagacgg |
| hemA_F_for | ggggctagctgtaaccacataaaatatagcagctgg |
| hemA_R_rev | ctgggacttcctcattacacacacactattatcac |
| ashemZ_F   | ggggtcttctacgcagacgtcagctggacag |
| ashemZ_R   | tgcagacttgcagctgtaaacacgctctctcttac |
| sirA_for   | tgagactgctgtaacactttggtgaacagc |
| sirA_rev   | gaaacgggaggacattatatg |
| cbiX_for   | aagatactagtgaaacgggagagcattag |
| cbiX_rev   | acacccggcgtattgctgaggcccgc |
| fnr_for    | tcaagatttcttctgctgacaalc |
| fnr_rev    | gtaaggactgtctttgctgcc |
| methF_for  | tcaagtacagtctgggagtggcgtc |
| methF_rev  | gaagtcgacgctctcgccagcagctgtaaagt |
| glmS_for   | tcaagtactagtgaaacgggagagcattag |
| glmS_rev   | gaaactttgcttctgctgacaalc |
| rtpR_for   | ggaagtcactttggtggtgcagtt |
| rtpR_rev   | gaagtcgacgctctcgccagcagctgtaaagt |

Restriction sites are shown in italic letters.

Cultivation conditions

Aerobic cultivations of pre- and main-cultures were in 50 ml shake flask cultures of Luria–Bertani (LB) medium (Sambrook and Russell, 2001) using a waterbath shaker (Aquatron, Infors AG, Bottmingen, Switzerland) at 250 r.p.m. at 30°C (autonomously replicating integratable plasmids), 37°C (autonomously replicating plasmids) or 42°C (integrated plasmids). Anaerobic conditions were achieved using 150 ml anaerobic flasks shaking at 100 r.p.m. at 30°C, 37°C or 42°C as outlined before (Reents et al., 2006b). Ten μg l−1 tetracycline (pMM1522- or pWH1520-derivatives), 5 μg l−1 erythromycin (pHBinTE-derivatives) or 0.4 μg l−1 kanamycin (pHBinNT-derivatives) were added to cultures carrying the different plasmids. Chromosomal integration processes of plasmid DNA was performed as described before (Barg et al., 2005). Chromosomally integrated Pbesot was induced by the addition of 0.23% (w/v) xylose to the growth medium. Expression of recombinant genes localized on plasmids was induced by the addition of 0.5% (w/v) xylose.

Analysis procedures

Samples for protein analysis and vitamin B12 measurement were taken as indicated time points. For analyzing the intracellular protein composition, cells were harvested by centrifugation and proteins were prepared and analyzed by SDS-PAGE gel as described before (Malten et al., 2005). To determine the intracellular vitamin B12 content, two different methods were used. The B. megaterium cells were harvested and disintegrated by a combined lyophilization and boiling processes. Cell extracts and bioassay plates for the final analysis were prepared, samples were spotted and incubated as described previously (Raux et al., 1996). Alternatively, the vitamin B12 content was measured using the RIDASCREENFAST Vitamin B12 ELISA test (r-biopharm; Germany) according to the manufacture’s introduction. All vitamin B12 measurements were done in triplicates with error margins of 10%.

Protoplast transformation of B. megaterium

Protoplasted B. megaterium cells were transformed with the corresponding expression plasmid using a PEG-mediated procedure (Barg et al., 2005).

Fluorescence spectroscopy

For fluorescence emission spectra, B. megaterium cells were harvested and suspended in ddH2O to achieve identical optical densities for all samples. Subsequently, the cells were destroyed with lysozyme (1 mg ml−1) treatment and sonication. After centrifugation, the supernatant was used for fluorescence measurements (Luminescense Spectrometer LS50B, PerkinElmer, Boston, MA) to detect coproporphyrinogen III and protoporphyrin IX. For detection of coproporphyrinogen...
Ill and protoporphyrinogen III, both were completely oxidized to coproporphyrin III and protoporphyrin IX, respectively, with 30% H$_2$O$_2$. At an excitation of 409 nm, coproporphyrin III shows two emission maxima at 590 and 620 nm while protoporphyrin IX one at 633 nm as outlined before (Layer et al., 2002).

Acknowledgements

We gratefully acknowledge financial support granted by the ‘Deutsche Forschungsgemeinschaft’, especially ‘Sonderforschungsbereich 578’, the ‘Fonds der Chemischen Industrie’ and the Biotechnology and Biological Sciences Research Council. Furthermore, we thank Wolfgang Hillen (Erlangen, Germany) for kindly providing the _B. megaterium_ expression and integration vectors and Wolfgang Buckel (Marburg, Germany) for pOZ3.

References

Andersson, D.I., and Roth, J.R. (1989) Redox regulation of the genes for cobinamide biosynthesis in _Salmonella typhimurium_. _J Bacteriol_ 171: 6734–6739.

Barg, H., Malten, M., Jahn, M., and Jahn, D. (2005) Protein and vitamin production in _Bacillus megaterium_. In _Microbial Processes and Products_. Vol. 18. Barredo, J.L. (ed.). Totowa, NJ, USA: Humana Press, pp. 165–184.

Bäumchen, C., Roth, A.H., Biedendieck, R., Malten, M., Fallmann, M., Sahm, H., et al. (2007) D-manitol production by resting state whole cell biotransformation of D-fructose by heterologous mannitol and formate dehydrogenase gene expression in _Bacillus megaterium_. _Biotechnol J_ 2: 1408–1416.

Beine, R., Moraru, N., Nimtz, M., Na'ammieh, S., Pawlowski, A., Buchholz, K., and Seibel, J. (2008) Synthesis of novel fructooligosaccharides by substrate and enzyme engineering. _J Biotechnol_ 138: 33–41.

Biedendieck, R., Yang, Y., Deckwer, W.D., Malten, M., and Jahn, D. (2007a) Plasmid system for the intracellular production and purification of affinity-tagged proteins in _Bacillus megaterium_. _Biotechnol Bioeng_ 96: 525–537.

Biedendieck, R., Beine, R., Garner, M., Jordan, E., Buchholz, K., Seibel, J., et al. (2007b) Export, purification, and activities of affinity tagged _Lactobacillus reuteri_ levanusucrase produced by _Bacillus megaterium_. _Appl Microbiol Biotechnol_ 74: 1062–1073.

Blanche, F., Thibaut, D., Debussche, L., Hertle, R., Zipfel, F., and Müller, G. (1993) Parallels and decisive differences in vitamin B$_12$ synthesis and vitamin B$_12$. In _Metabolic engineering of vitamin B$_{12}$ production in _B. megaterium_. Biochem Biophys Res Commun_ 307: 584–588.

Chen, D., Abend, A., Stubbe, J., and Frey, P.A. (2003) Epimerization at carbon-5‘ of (5‘R)-[5‘-2H] adenosylcobalamin by ribonucleoside triphosphate reductase: cysteine 408-independent cleavage of the Co-C5‘ bond. _Biochemistry_ 42: 4578–4584.

Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., and Smith, A.G. (2005) Algae acquire vitamin B$_{12}$ through a symbiotic relationship with bacteria. _Nature_ 438: 90–93.

Cruz Ramos, H., Hoffmann, T., Marino, M., Nedjari, H., Presecan-Siedel, E., Dreessen, O., et al. (2000) Fermentative metabolism of _Bacillus subtilis_: physiology and regulation of gene expression. _J Bacteriol_ 182: 3072–3080.

Darie, S., and Gunsalus, R.P. (1994) Effect of heme and oxygen availability on _hemA_ gene expression in _Escherichia coli_: role of the _fnr_, _arcA_, and _himA_ gene products. _J Bacteriol_ 176: 5270–5276.

Doss, M., and Philipp-Dormston, W.K. (1971) Porphyrin and heme biosynthesis from endogenous and exogenous delta-aminolevulinic acid in _Escherichia coli_. _Pseudo- monas aeruginosa_, and _Achromobacter metalcaligenes_. _Hoppe Seylers Z Physiol Chem_ 352: 725–733.

Doss, M.O. (1974) Porphyrins and porphyrin precursors. In _Clinical Biochemistry. Principles and Methods_. Curtius, H., and Roth, M. (eds). New York, NY, USA: de Gruyter, 1323–1371.

Drennan, C.L., Huang, S., Drummond, J.T., Matthews, R.G., and Lidwig, M.L. (1994) How a protein binds B$_{12}$: a 3.0 Å X-ray structure of B$_{12}$-binding domains of methionine synthase. _Science_ 266: 1669–1674.

Eichmüller, C., Tolleringer, M., Kräutler, B., and Konrat, R. (2001) Mapping the ligand binding site at protein side-chains in protein-ligand complexes through NOE difference spectroscopy. _J Biomol NMR_ 20: 195–202.

Escalante-Semerena, J.C., and Warren, M.J. (2008) Biosynthesis and use of cobalamin (B$_{12}$) in _Escherichia coli_ & _Salmonella typhimurium: Cellular & Molecular Biology (Web-only module)_. Vol. 2008. Chapter 3.6.3.8. Böck, A.R.C., III, Kaper, J.B., Karp, P.D., Neidhardt, F.C., Nystrom, T., Slauch, J.M., and Squires, C.L. (eds). Washington, D.C., USA: ASM Press.

Eschenmoser, A., and Wintner, C.E. (1977) Natural product synthesis and vitamin B$_{12}$. _Science_ 196: 1410–1420.

Glick, B.R. (1995) Metabolic load and heterologous gene expression. _Biotechnol Adv_ 13: 247–261.

Grote, A., Hiller, K., Scheer, M., Münch, R., Nörtemann, B., Hempel, D.C., and Jahn, D. (2005) JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. _Nucleic Acids Res_ 33: W526–531.

Hall, D.A., Vander Kozi, C.W., Stasik, C.N., Stevens, S.Y., Zuiderviet, E.R., and Matthews, R.G. (2001) Mapping the interactions between flavodoxin and its physiological partners flavodoxin reductase and cobalamin-dependent methionine synthase. _Proc Natl Acad Sci USA_ 98: 9521–9526.

Hoffmann, T., Frankenberg, N., Marino, M., and Jahn, D. (1998) Ammonification in _Bacillus subtilis_ utilizing dissimilatory nitrite reductase is dependent on resDE. _J Bacteriol_ 180: 186–189.

© 2009 The Authors

Journal compilation © 2009 Society for Applied Microbiology and Blackwell Publishing Ltd, _Microbial Biotechnology_, 3, 24–37.
Homuth, G., Rompf, A., Schumann, W., and Jahn, D. (1999) Transcriptional control of *Bacillus subtilis* hemN and hemZ. *J Bacteriol* 181: 5922–5929.

Jeter, R.M., and Roth, J.R. (1987) Cobalamin (vitamin B<sub>12</sub>) biosynthetic genes of *Salmonella typhimurium*. *J Bacteriol* 169: 3189–3198.

Jones, R.M., and Jordan, P.M. (1993) Purification and properties of the uroporphyrinogen decarboxylase from *Rhodobacter sphaeroides*. *Biochim J* 293: 703–712.

Kang, S.H., Singh, S., Kim, J.Y., Lee, W., Mulchandani, A., and Chen, W. (2007) Bacteria metabolically engineered for enhanced phytochelatin production and cadmium accumulation. *Appl Environ Microbiol* 73: 6317–6320.

Layer, G., Verfurth, K., Mahlitz, E., and Jahn, D. (2002) Oxygen-dependent coproporphyrinogen-III oxidase HemN from *Escherichia coli*. *J Biol Chem* 277: 34196–34142.

Leech, H.K., Raux-Deey, E., Heathcote, P., and Warren, M.J. (2002) Production of cobalamin and sirohaem in *Bacillus megaterium*: an investigation into the role of the branch-point chelatases sirohydrochlorin ferrochelatase (SirB) and sirohydrochlorin cobalt chelatase (CbiX). *Biochem Soc Trans* 30: 610–613.

Luschinsky, C.L., Drummond, J.T., Matthews, R.G., and Ludwig, M.L. (1992) Crystallization and preliminary X-ray diffraction studies of the cobalamin-binding domain of methionine synthase from *Escherichia coli*. *J Mol Biol* 225: 557–560.

Malten, M., Hollmann, R., Deckwer, W.D., and Jahn, D. (2005) Production and secretion of recombinant *Leucochloridium aestuarii* dextranucrase DsrS in *Bacillus megaterium*. *Biotechnol Bioeng* 89: 206–218.

Malten, M., Biedendieck, R., Gamer, M., Drews, A.C., Stammen, S., Buchholz, K., et al. (2006) A *Bacillus megaterium* plasmid system for the production, export, and one-step purification of affinity-tagged heterologous levanucrase from growth medium. *Appl Environ Microbiol* 72: 1677–1679.

Mancia, F., Keep, N.H., Nakagawa, A., Leadlay, P.F., McSweeney, S., Rasmussen, B., et al. (1996) How coenzyme B<sub>12</sub> radicals are generated: the crystal structure of methylmalonyl-coenzyme A mutase at 2 Å resolution. *Structure* 4: 339–350.

Marino, M., Ramos, H.C., Hoffmann, T., Glaser, P., and Jahn, D. (2001) Modulation of anaerobic energy metabolism of *Bacillus subtilis* by arfM (ywiD). *J Bacteriol* 183: 6815–6821.

Martens, J.H., Barg, H., Warren, M.J., and Jahn, D. (2002) Microbial production of vitamin B<sub>12</sub>. *Appl Microbiol Biotechnol* 58: 275–285.

Moser, J., Schubert, W.D., Heinz, D.W., and Jahn, D. (2002) Structure and function of glutamyl-tRNA reductase involved in 5-amino-levulinic acid formation. *Biochem Soc Trans* 30: 579–584.

Münch, R., Hiller, K., Grote, A., Scheer, M., Klein, J., Schober, M., and Jahn, D. (2005) Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* 21: 4187–4189.

Nakahashi, K., Nishimura, K., Miyamoto, K., and Inokuchi, H. (1991) Photosensitivity of a protoporphyrin-accumulating, light-sensitive mutant (visA) of *Escherichia coli* K-12. *Proc Natl Acad Sci USA* 88: 10520–10524.

Raux, E., Lanois, A., Levillayer, F., Warren, M.J., Brody, E., Rambach, A., and Therme, C. (1996) *Salmonella typhimurium* cobalamin (vitamin B<sub>12</sub>) biosynthetic genes: functional studies in *S. typhimurium* and *Escherichia coli*. *J Bacteriol* 178: 753–767.

Raux, E., Lanois, A., Warren, M.J., Rambach, A., and Therme, C. (1998a) Cobalamin (vitamin B<sub>12</sub>) biosynthesis: identification and characterization of a *Bacillus megaterium* cobl operon. *Biochem J* 335: 159–166.

Raux, E., Lanois, A., Rambach, A., Warren, M.J., and Therme, C. (1998b) Cobalamin (vitamin B<sub>12</sub>) biosynthesis: functional characterization of the *Bacillus megaterium* cbi genes required to convert uroporphyrinogen III into cobyrinic acid a,c-diamide. *Biochem J* 335: 167–173.

Raux, E., Schubert, H.L., and Warren, M.J. (2000) Biosynthesis of cobalamin (vitamin B<sub>12</sub>): a bacterial conundrum. *Cell Mol Life Sci* 57: 1880–1893.

Raux, E., Leech, H.K., Beck, R., Schubert, H.L., Santander, P.J., Roessner, C.A., et al. (2003) Identification and functional analysis of enzymes required for precorrin-2 dehydrogenation and metal ion insertion in the biosynthesis of sirohaem and cobalamin in *Bacillus megaterium*. *Biochem J* 370: 505–516.

Ravnum, S., and Andersson, D.I. (2001) An adenosyl-cobalamin (coenzyme-B<sub>12</sub>)-repressed translational enhancer in the cob mRNA of *Salmonella typhimurium*. *Mol Microbiol* 39: 1585–1594.

Reents, H., Münch, R., Damtey, T., Jahn, D., and Hartig, E. (2006a) The Fnr regulon of *Bacillus subtilis*. *J Bacteriol* 188: 1103–1112.

Reents, H., Gruner, I., Harmening, U., Böttger, L.H., Layer, G., Heathcote, P., et al. (2006b) *Bacillus subtilis* Fnr senses oxygen via a [4Fe-4S] cluster coordinated by three cysteine residues without change in the oligomeric state. *Mol Microbiol* 60: 1432–1445.

Robin, C., Blanche, F., Cauchois, L., Cameron, B., Couder, M., and Crouzet, J. (1991) Primary structure, expression in *Escherichia coli*, and properties of S-Adenosyl-L-methionine-uroorphyrinogen III methyltransferase from *Bacillus megaterium*. *J Bacteriol* 173: 4893–4896.

Roth, J.R., Lawrence, J.G., Rubenfield, M., Kieffer-Higgins, S., and Church, G.M. (1993) Characterization of the cobalamin (vitamin B<sub>12</sub>) biosynthetic genes: functional studies in *Salmonella typhimurium*. *J Bacteriol* 175: 3303–3016.

Roth, J.R., Lawrence, J.G., and Bobik, T.A. (1996) Cobalamin (coenzyme B<sub>12</sub>): synthesis and biological significance. *Annu Rev Microbiol* 50: 137–181.

Rygus, T., and Hillen, W. (1991) Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. *Appl Microbiol Biotechnol* 35: 594–599.

Rygus, T., Scheler, A., Allmansberger, R., and Hillen, W. (1991) Molecular cloning, structure, promoters and regulatory elements for transcription of the *Bacillus megaterium* encoded regulon for xylose utilization. *Arch Microbiol* 155: 535–542.

Sambrook, J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
Schmiedel, D., Vary, P.S., Jablonski, L., and Hillen, W. (1997) Plasmids for efficient single-copy gene cloning into \textit{gdh2} and \textit{trpC} of \textit{Bacillus megaterium} DSM319 and QM B1551. \textit{Appl Microbiol Biotechnol} \textbf{47}: 543–546.  

Schobert, M., and Jahn, D. (2002) Regulation of heme biosynthesis in non-phototrophic bacteria. \textit{J Mol Microbiol Biotechnol} \textbf{4}: 287–294.  

Shen, C.R., and Liao, J.C. (2008) Metabolic engineering of \textit{Escherichia coli} for 1-butanol and 1-propanol production via the keto-acid pathways. \textit{Appl Microbiol Biotechnol} \textbf{47}: 543–546.  

Shrader, T.E., Tobias, J.W., and Varshavsky, A. (1993) The N-end rule in \textit{Escherichia coli}: cloning and analysis of the leucyl, phenylalanyl-tRNA-protein transferase gene \textit{aat}. \textit{J Bacteriol} \textbf{175}: 4364–4374.  

Spizizen, J. (1958) Transformation of biochemically deficient strains of \textit{Bacillus subtilis} by deoxyribonucleate. \textit{Proc Natl Acad Sci USA} \textbf{44}: 1072–1078.  

Tatarko, M., and Romeo, T. (2001) Disruption of a global regulatory gene to enhance central carbon flux into phenylalanine biosynthesis in \textit{Escherichia coli}. \textit{Curr Microbiol} \textbf{43}: 26–32.  

Thibaut, D., Blanche, F., Cameron, B., Crouzet, J., Debussche, L., Remy, E., and Vuilhorgne, M. (1998) Vitamin B\textsubscript{12} biosynthesis in \textit{Pseudomonas aeruginosa}. In \textit{Vitamin B\textsubscript{12} and \textit{B}12-Proteins}. Kräutler, B., Arigoni, D., and Golding, B.T. (eds). Weinheim, Germany: Wiley/VCH, pp. 63–79.  

Vary, P.S. (1992) Development of genetic engineering in \textit{Bacillus megaterium}. In \textit{Biology of Bacilli: Application to Industry}. Doi, R., and McGloughlin, M. (eds). Boston, MA, USA: Heinemann-Butterworths, pp. 251–310.  

Vary, P.S. (1994) Prime time for \textit{Bacillus megaterium}. \textit{Microbiology} \textbf{140}: 1001–1013.  

Vary, P.S., Biedendieck, R., Fuerch, T., Meinhardt, F., Rohde, M., Deckwer, W.D., and Jahn, D. (2007) \textit{Bacillus megaterium}—from simple soil bacterium to industrial protein production host. \textit{Appl Microbiol Biotechnol} \textbf{76}: 957–967.  

Wang, L., Wilson, S., and Elliott, T. (1999) A mutant HemA protein with positive charge close to the N-terminus is stabilized against heme-regulated proteolysis in \textit{Salmonella typhimurium}. \textit{J Bacteriol} \textbf{181}: 603–6041.  

Winstedt, L., and von Wachenfeldt, C. (2000) Terminal oxidases of \textit{Bacillus subtilis} strain 168: one quinol oxidase, cytochrome \textit{aa3}, or cytochrome \textit{bd}, is required for aerobic growth. \textit{J Bacteriol} \textbf{182}: 6557–6564.  

Wolf, J.B., and Brey, R.N. (1986) Isolation and genetic characterizations of \textit{Bacillus megaterium} cobalamin biosynthesis-deficient mutants. \textit{J Bacteriol} \textbf{166}: 51–58.  

Woodward, R.B. (1973) The total synthesis of vitamin B\textsubscript{12}. \textit{Pure Appl Chem} \textbf{33}: 145–177.  

Yakandawala, N., Romeo, T., Friesen, A.D., and Madhyastha, S. (2008) Metabolic engineering of \textit{Escherichia coli} to enhance phenylalanine production. \textit{Appl Microbiol Biotechnol} \textbf{78}: 283–291.  

Yang, Y., Biedendieck, R., Wang, W., Gamer, M., Malten, M., Jahn, D., and Deckwer, W.D. (2006) High yield recombinant penicillin G amidase production and export into the growth medium using \textit{Bacillus megaterium}. \textit{Microb Cell Fact} \textbf{5}: 36.  

Yang, Y., Malten, M., Grote, A., Jahn, D., and Deckwer, W.D. (2007) Codon optimised \textit{Thermobifida fusca} hydrolase secreted by \textit{Bacillus megaterium}. \textit{Biotechnol Bioeng} \textbf{96}: 780–794.  

Zadra, I., Abt, B., Parson, W., and Haas, H. (2000) \textit{xylIP} promoter-based expression system and its use for antisense downregulation of the \textit{Penicillium chrysogenum} nitrogen regulator NRE. \textit{Appl Environ Microbiol} \textbf{66}: 4810–4816.  

Zelder, O., Beatrix, B., and Buckel, W. (1994) Cloning, sequencing and expression in \textit{Escherichia coli} of the gene encoding component S of the coenzyme B\textsubscript{12}-dependent glutamate mutase from \textit{Clostridium cochlearium}. \textit{FEMS Microbiol Lett} \textbf{118}: 15–21.