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Metabolic engineering of cobalamin (vitamin B_{12}) production in *Bacillus megaterium*

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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 B_{12} biosynthesis, including *cbiX*, *sirA*, modified *hemA*, the operons *hemAXCDBL* and *cbiXJCDETLFGAcysG*^{A}*cbiYbtuR*, 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 *cbiXJCDETLFGAcysG*^{A}*cbiYbtuR* 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.

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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

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more than 60 steps (Woodward, 1973; Eschenmoser and Wintner, 1977). For this reason, commercial production of vitamin B_{12} 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 coenzyme 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 adenosylcobyric 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 B_{12} 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 (Rygus and Hillen, 1991; Rygus *et al.*, 1991; Biedendieck *et al.*, 2007a), whereby the P_{xylA} 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 B_{12} 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 (protoheme), the protein itself is subject to protoheme-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 hemA$_{KK}$* gene was cloned downstream of the xylose-inducible promoter P_{xylA} in the integrative plasmid pHBintE (Fig. 2AII). This led to the generation of plasmid pHBihemA$_{KK}$, 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 HemA$_{KK}$, which was produced at levels detectable by SDS-PAGE analysis (data not shown). The effect of this increased recombinant HemA$_{KK}$ production resulted in a 13.5-fold (10.8-fold) rise in the intracellular vitamin B_{12} concentration to 0.54 $\mu g\ l^{-1}\ OD_{578nm}{}^{-1}$

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MT--LLALGINHKTAPVSLRERVTFSP   S.typhimurium HemA
MH--IIAVGLNFRTAPVEIREKLSFNE   B.megaterium HemA
MHKKIIAVGLNFRTAPVEIREKLSFNE   B.megaterium HemAKK
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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.

(2.81 μg l^{-1}) (Table 1) in comparison with the wild-type strain (0.04 μg l^{-1} OD_{578nm}^{-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 pHBihemA$_{KK}$ to 42°C resulting in the construction of strain HBBm1 (Fig. 2A). As a consequence, the expression of the complete chromosomal *B. megaterium hemA*$_{KK}$*XCDBL* operon was brought under the control of the strong inducible promoter P_{xylA}. 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 B_{12} to 1.59 μg l^{-1} OD_{578nm}^{-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 *hemA*$_{KK}$*XCDBL* operon was eliminated. This problem was overcome by growing the strain at 42°C.

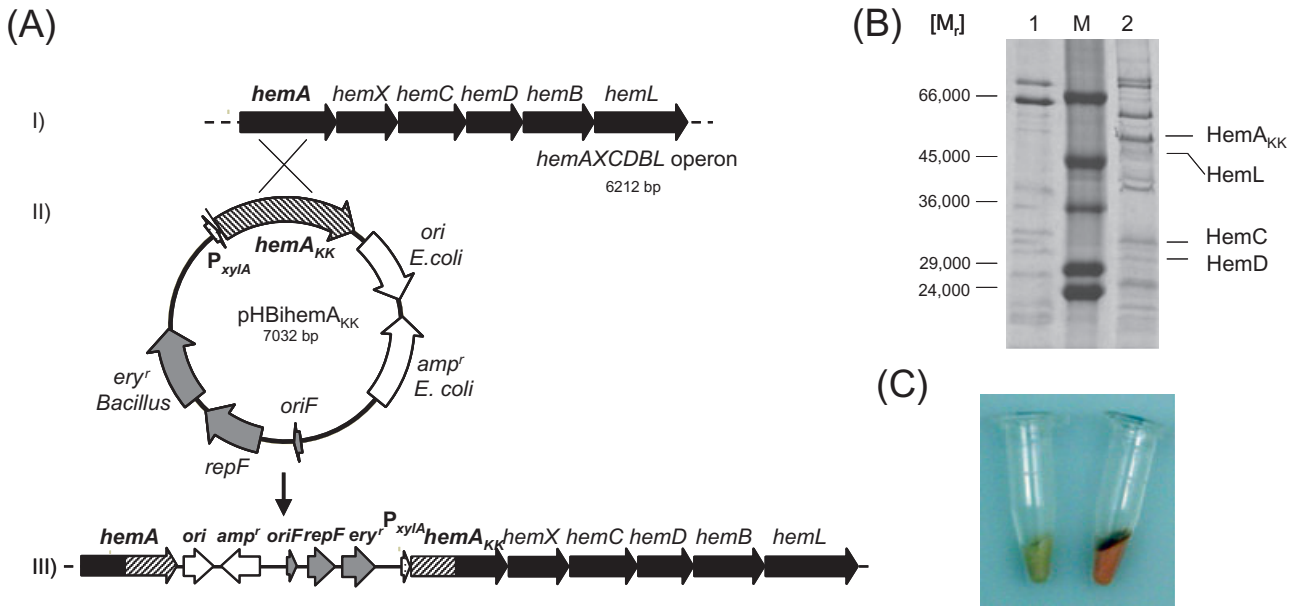

Fig. 2. Molecular strategy for the overexpression of the chromosomal *hemA*$_{KK}$*XCDBL* operon.
A. After construction of the integrative plasmid pHBihemA$_{KK}$ (II), the plasmid was integrated into the *B. megaterium* chromosome (I) via a single cross-over recombination (III). Plasmid elements of pHBihemA$_{KK}$ are the xylose-inducible promoter P_{xylA}, the temperature-sensitive origin of plasmid replication *oriF*, the gene essential for plasmid replication *repF* and the gene responsible for erythromycin resistance *ery*^{r}. Plasmid elements for plasmid replication in *E. coli* are the origin of replication *colE1* and the *β-lactamase* gene *amp*^{r} 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*$_{KK}$*XCDBL* operon. The strong formation of tetrapyrroles is indicated by the red colour.

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Table 1. Summary of intracellular vitamin B_{12} amounts for the various optimization experiments with *B. megaterium*.

B. megaterium strain	Relevant genotype	Strategy	Description	Vitamin B_{12} $\mu g\ l^{-1} OD^{-1}_{578nm}$	Factor	Vitamin B_{12} $\mu g\ l^{-1}$	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-pHBiHemA$_{KK}$	P_{xylA} *hemA$_{KK}$* (plasmid)	↑	Overexpression of feedback deregulated HemA	0.54	13.5	2.81	10.8
HBBm1	P_{xylA} *hemA$_{KK}$XCDBL* (genome)	↑	Overexpression of chromosomal *hemA$_{KK}$XCDBL* operon and feedback deregulation of HemA	1.59	39.8	8.51	32.7
DSM509-pWH1520sirA	P_{xylA} *sir* (plasmid)	↑	Overexpression of *sirA*	0.18	4.5	1.10	4.2
DSM509-pHBicbiX	P_{xylA} *cbiX* (plasmid)	↑	Overexpression of *cbiX*	0.35	8.8	0.60	2.3
DSM509-pHBicbiX (cobalt addition)	P_{xylA} *cbiX* (plasmid)	↑	Overexpression of *cbiX* (cobalt addition)	0.82	20.5	1.59	6.1
HBBm3	P_{xylA} *cbiXJCDETLF GAcysG^{A}cbiYbtuR* (genome)	↑	Overexpression of chromosomal *cobI* operon	0.47	11.8	0.85	3.5
HBBm3 (cobalt addition)	P_{xylA} *cbiXJCDETLF GAcysG^{A}cbiYbtuR* (genome)	↑	Overexpression of chromosomal *cobI* operon (cobalt addition)	0.79	19.8	1.24	4.8
DSM509-pWH1520ashemZ	P_{xylA} *ashemZ* (plasmid)	↓	Downregulation of heme via *antisense hemZ* RNA	0.05	1.2	0.31	1.2
DSM509-pWH1520fnr	P_{xylA} *fnr$_{Bsub}$* (plasmid)	↑	Overexpression of *fnr*	0.19	4.8	0.97	3.7
HBBm1-pWH1520ashemZ (3 h/5 h after induction)	P_{xylA} *hemA$_{KK}$XCDBL* (genome) P_{xylA} *ashemZ* (plasmid)	↑ & ↓	Overexpression of chromosomal *hemA$_{KK}$XCDBL* operon, feedback deregulation of HemA and downregulation of heme via *antisense hemZ* RNA	1.32/0.92	33.0/22.9	4.50/3.53	17.3/13.6
HBBm1-pWH1520sirA (3 h/5 h after induction)	P_{xylA} *hemA$_{KK}$XCDBL* (genome) P_{xylA} *sirA* (plasmid)	↑ & ↑	Overexpression of chromosomal *hemA$_{KK}$XCDBL* 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*	P_{xylA} *metH** (plasmid)	↑	Overexpression of *metH** and feedback deregulation	0.06	1.5	0.41	1.6
DSM509-pMM1522rtpR	P_{xylA} *rtpR* (plasmid)	↑	Overexpression of *rtpR* and feedback deregulation	0.19	4.7	1.14	4.4
DSM509-pMM1522glmS	P_{xylA} *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 B_{12} concentrations per cell expressed as $\mu g\ l^{-1}\ OD^{-1}_{578nm}$, the volumetric amount of B_{12} as $\mu g\ l^{-1}$, as well as the incremental factors related to vitamin B_{12} 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 B_{12} measurements were done in triplicates with error margins of 10%.

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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,

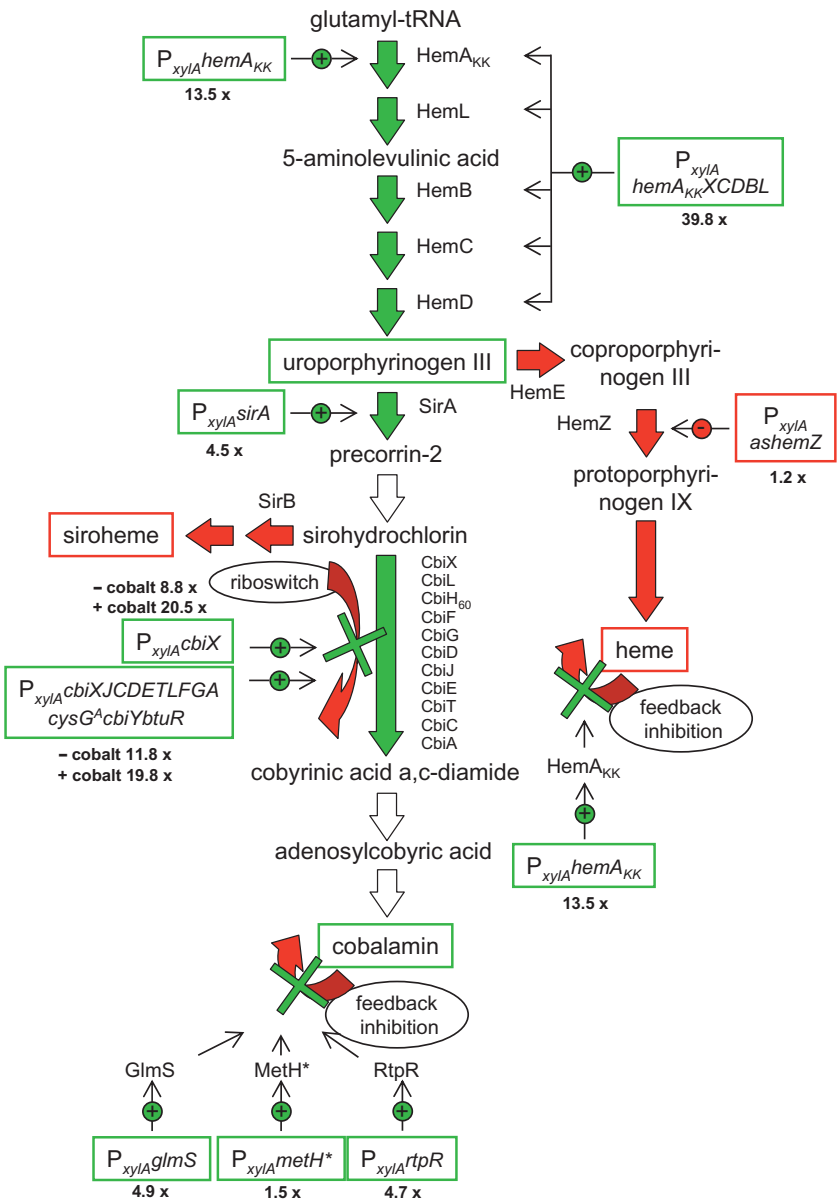

Fig. 3. Optimization strategies for biosynthesis of vitamin B_{12} in *B. megaterium*. The biosynthesis of vitamin B_{12} and heme with the relevant steps to our approach is shown. Green arrows show the flow of intermediates to vitamin B_{12} while red arrows indicate the competing pathways for heme 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_{xylA}' 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. $hemA_{KK}$/$HemA_{KK}$ – 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*.

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a 129 bp long DNA sequence coding for an *antisense hemZ* RNA (*ashemZ*) was designed and placed under the control of P_{xylA}, creating plasmid pWH1520ashemZ. The asRNA included 82 nt upstream of the start codon of *hemZ* as well as the first 47 nt of *hemZ* to allow complete hybridization with the ribosomal binding site of the *hemZ* mRNA. The *ashemZ* : *hemZ* mRNA duplex renders the ribosome binding site inaccessible for the ribosome (Zadra *et al*., 2000) and thereby prevents *hemZ* mRNA translation.

To evaluate the physiological consequences of *ashemZ* expression, we determined the coproporphyrinogen III : protoporphyrinogen IX as well as the heme and vitamin B_{12} ratio 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_{12} concentration by 20% (0.05 μg l^{-1} OD_{578nm}^{-1} and 0.31 μg l^{-1}, 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_{12} 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_{12} 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 transmethylations 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_{12} biosynthesis, the *sirA* gene was overexpressed from an autonomously replicating plasmid under the control of P_{xylA} (pWH1520sirA). This approach resulted in a 4.5- and 4.2-fold increase (0.18 μg l^{-1} OD_{578nm}^{-1} and 1.1 μg l^{-1}) in the intracellular vitamin B_{12} 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_{12} biosynthesis*

Cobalt is the central ion of all cobalamins. It is a trace element and its concentration in the growth medium may represent a limiting factor for vitamin B_{12} 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^{-1} OD_{578nm}^{-1}) and 2.2-fold (0.56 μg l^{-1}) 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_{12} (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 sirohydrochlorin making 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_{12} production in *B. megaterium.* This operon encodes *cbiW, cbi*H_{60}*, cbiX, cbiJ, cbiC, cbiD, cbiET, cbiL, cbiF, cbiG, cbiA, cysG*^{A}*, cbiY* and *btuR* necessary for cobyrinic acid a,c-diamide formation (Raux *et al*., 1998b). Furthermore, in *S. enterica*, the end-

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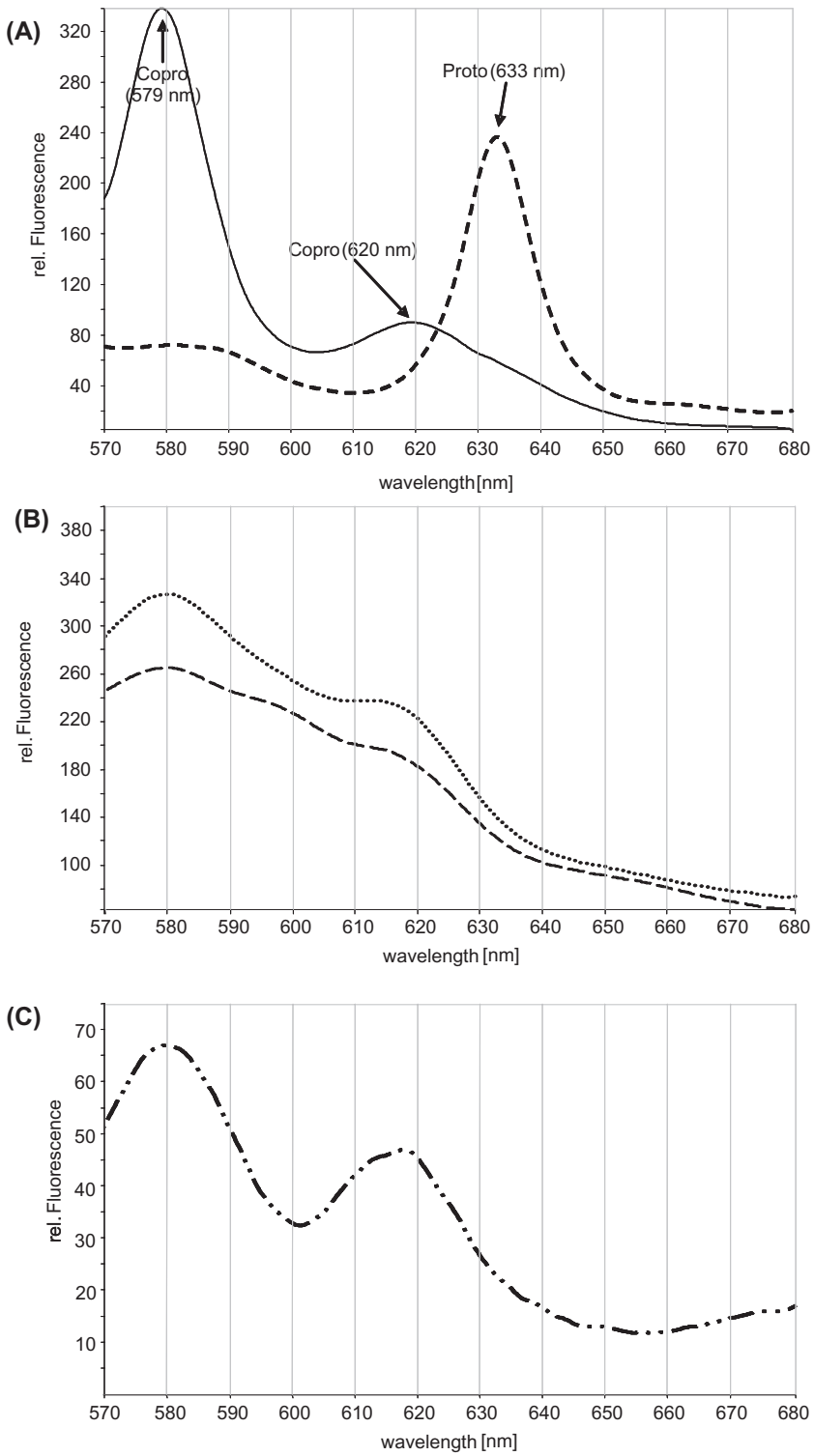

Fig. 4. Fluorescence spectra of *B. megaterium* cell extracts for relative quantification of coproporphyrinogen III and protoporphyrinogen IX. After cultivation, cells were harvested and treaded as described in *Experimental procedures*. The relative fluorescence of the cell extracts was measured with an excitation wavelength of 409 nm and an emission wavelength from 570 to 680 nm. Fluorescence spectra were obtained for: (A) standard curves with standard solutions of coproporphyrin III (black line) and protoporphyrin IX (dotted line); (B) cell-free extracts of *B. megaterium* DSM509 transformed with pWH1520ashemZ (dotted line) and DSM509 transformed with pWH1520 (dashed line) after oxidation with 30% hydrogen peroxide; (C) difference spectrum of the two spectra of (B).

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 pHBintN to construct pHBicbiX. This autonomously replicating plasmid mediated xylose-

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inducible overexpression of *cbiX* (P_{xylA}-*cbiX*) after it was transferred into *B. megaterium* strain DSM509, which resulted in significantly increased intracellular CbiX levels (data not shown). In contrast to wild-type *B. megaterium*, 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 *B. megaterium* DSM509 transformed with pHBicbiX were 8.8- (0.35 μg l^{-1} OD_{578nm}^{-1}) and 20.5-fold (0.82 μg l^{-1} OD_{578nm}^{-1}) 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_{12} concentrations were enhanced 2.3- (0.6 μg l^{-1}) and 6.1-fold (1.59 μg l^{-1}), 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_{xylA} 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 $cbiH_{60}$, remained under the control of the natural promoter of the operon, but the subsequent genes now come under the control of P_{xylA}. 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_{12} concentrations [0.47 μg l^{-1} OD_{578nm}^{-1} (0.85 μg l^{-1}) without and 0.79 μg l^{-1} OD_{578nm}^{-1} (1.24 μg l^{-1}) 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 *B. megaterium* houses an oxygen-independent cobalamin biosynthetic pathway (Raux *et al.*, 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 *B. megaterium* had a 5-fold induction of cobalamin production (0.2 μg l^{-1} OD_{578nm}^{-1}) when grown anaerobically. Similar observations have also been made with cobalamin formation in *S. enterica* (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_{12} concentration to 0.08 μg l^{-1} compared with 0.26 μg l^{-1} for the aerobic cultivation conditions (Table 1).

In *B. subtilis*, the model organism for Gram-positive *bacilli*, the oxygen global regulator Fnr mediates anaerobic gene expression (Marino *et al.*, 2001; Reents *et al.*, 2006a,b). In order to combine aerobic growth yields with the efficiency of anaerobic vitamin B_{12} production, *B. subtilis fnr* was recombinantly overexpressed in aerobically grown *B. megaterium* DSM509 by cloning the gene under the control of P_{xylA}. *B. megaterium* transformed with pWH1520fnr showed a 4.8-fold increase in the amount of cobalamin per cell (0.19 μg l^{-1} OD_{578nm}^{-1}) and significantly a 3.7-fold increase in the volumetric cobalamin amount (0.97 μg l^{-1}) (Table 1). Aerobically produced *B. subtilis* 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 *B. megaterium* genome offered the opportunity to screen for potential Fnr binding sites using the Virtual Footprint tool and the PRODORIC database (Münch *et al.*, 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 *B. subtilis* 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 *et al.*, 1998). Both activities are also integral parts of the aerobic nitrogen assimilation and carbon overflow metabolism (Cruz Ramos *et al.*, 2000). In *B. subtilis*, the quinol oxidase operon *qoxABCD* encodes the major oxygen-dependent terminal oxidase cytochrome caa_3 (Winstedt and von Wachenfeldt, 2000). In *B. megaterium*, Fnr-dependent additional induction of this system under aerobic conditions might also contribute to the overall ATP production, growth yield and consequently vitamin B_{12} biosynthesis.

Overproduction of vitamin B_{12} 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_{12} binding domains were selected. These included the vitamin B_{12} binding

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domain of *E. coli* methionine synthase MetH (further named MetH*, $M_r = 28\,000$), which was shown to bind vitamin B_{12} with a K_d of 1.2 μM, (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_r of 14 800 and a K_d (GlmS/vitamin B_{12}) of 5.6 ± 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 μM (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_{xylA} control into pMM1522 and tested for recombinant overexpression in *B. megaterium* DSM509. Recombinant overexpression of *metH** resulted in a 1.5-fold (1.6-fold) increase in the intracellular cobalamin level to 0.06 μg l^{-1} OD_{578nm}^{-1} (0.41 μg l^{-1}) while overexpression of *rtpR* and *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 μg l^{-1} OD_{578nm}^{-1} (1.14 and 1.2 μg 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 ± 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 *glmS* gene, the values of 0.2 and 0.22 obtained for *rptR* and *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 $hemA_{KK}XCDBL$ 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 μg l^{-1} OD_{578nm}^{-1} h^{-1}) was found within the first 2 h compared with that of *B. megaterium* HBBm1 without the *ashemZ* plasmid (0.23 μg l^{-1} OD_{578nm}^{-1} h^{-1}). Two hours after induction of expression of the recombinant gene, a cobalamin concentration of 1.32 μg l^{-1} OD_{578nm}^{-1} (4.5 μg l^{-1}) was measured. However, 5 h after induction, the concentration decreased again to 0.92 μg l^{-1} OD_{578nm}^{-1} (3.53 μg 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 $hemA_{KK}XCDBL$ operon and the *sirA* gene, both placed under the control of the xylose-inducible promoter P_{xylA}, were combined to enhance the biosynthetic steps leading to sirohydrochlorin (Fig. 3). For this purpose, HBBm1 carrying P_{xylA} upstream of the chromosomal $hemA_{KK}XCDBL$ 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 μg l^{-1} OD_{578nm}^{-1} h^{-1}, respectively) with a cobalamin concentration of 1.17 μg l^{-1} OD_{578nm}^{-1} (3.52 μg l^{-1}) (Table 1). Nevertheless, the maximal cobalamin concentrations of 1.49 and 1.59 μg l^{-1} OD_{578nm}^{-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 μg 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 $hemA_{KK}XCDBL$ 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

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Table 2. Strains used in this study.

Name	Description	Reference or source
E. coli		
DH10B	F⁻*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*K λ⁻*rps*L *nup*G	Gibco Life Technologies
B. megaterium		
DSM509	Wild-type strain, vitamin B_{12} producer	DSMZ*, Braunschweig, Germany
HBBm1	Mutant of DSM509, chromosomal overexpression of the *hemA*$_{KK}$*XCDBL* operon under xylose controlled P_{xylA}	This work
HBBm3	Mutant of DSM509, chromosomal overexpression of the *cbiX* gene and the downstream located genes of the *cobI* operon under xylose controlled P_{xylA}	This work
B. subtilis		
168	Wild-type strain	Spizizen (1958)
L. delbrueckii ssp. lactis		
DSM20076	Wild-type strain, vitamin B_{12} producer	DSMZ*, Braunschweig, Germany

*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$_{KK}$_for and hemA$_{KK}$_rev and ligated

Table 3. Plasmids used in this study.

Name	Description	Reference or source
pWH1520	Shuttle vector for cloning in *E. coli* (Ap^r) and recombinant gene expression under xylose control in *B. megaterium* (Tc^r)	Rygus and Hillen (1991)
pMM1522	Shuttle vector for cloning in *E. coli* (Ap^r) and recombinant gene expression under xylose control in *B. megaterium* (Tc^r); P_{xylA}-MCS	Malten *et al.* (2006)
pWH1967K	Cloning, expression and integration vector for *Bacillus spp.*; Ap^r, Tc^r, Nm^r, *ori*^{ts}	Schmiedel *et al.* (1997)
pHBintE	Cloning, expression and integration vector for *Bacillus spp.* with xylose-inducible promoter P_{xylA}; Ap^r, Em^r, *ori*^{ts}	Barg *et al.* (2005)
pHBintT	Cloning, expression and integration vector for *Bacillus spp.* with xylose-inducible promoter P_{xylA}; Ap^r, Tc^r, *ori*^{ts}	This work
pHBintN	Cloning, expression and integration vector for *Bacillus spp.* with xylose-inducible promoter P_{xylA}; Ap^r, Nm^r, *ori*^{ts}	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)
pHBihemA$_{KK}$	pHBintE-derivative: vector for chromosomal integration of the xylose-inducible promoter P_{xylA} downstream of the *B. megaterium hemA*$_{KK}$*XCDBL* 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
pHBicbiX	pHBintN-derivative: vector for chromosomal integration of the xylose-inducible promoter P_{xylA} downstream of the *cbiX* gene in the *cobI* operon of *B. megaterium*	This work
pWH1520fnr	pWH1520-derivative: vector for recombinant overexpression of *B. subtilis fnr* in *B. megaterium*	This work
pMM1522metH*	pMM1522-derivative: vector for recombinant overexpression of *E. coli metH** in *B. megaterium*	This work
pMM1522glmS	pMM1522-derivative: vector for recombinant overexpression of *C. cochlearium glmS* in *B. megaterium*	This work
pMM1522rtpR	pMM1522-derivative: vector for recombinant overexpression of *L. delbrueckii* ssp. *lactis rtpR* in *B. megaterium*	This work

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Table 4. Oligos used in this study.

Name	Sequence 5′-3′
intN_for	ggat*ccatgg*gtcgactctagagcttggg
intN_rev	ccac*aagctt*gtcatcgttcaaaatggtatgcg
intT_for	aag*catgcat*cagggttattgtctcatgagcgg
intT_rev	aaaa*aagctt*ccttgatgacacagaagaaggcg
$hemA_{KK}$_for	gggg*actagt*caaatgcataaaaaaattatagcagtcgg
$hemA_{KK}$_rev	ctgg*ggtacc*ccatatcaaccattattcaatcc
ashemZ_for	gcg*ggatcc*cttgaactgagcaccttgaccgg
ashemZ_rev	tcg*actagt*cggacgtaaaaaacgttcatcttctatacc
sirA_for	tcagt*actagt*catatggggaaagtatatvtagtcg
sirA_rev	gaagtc*ggatcc*gtaagcttcttgtgcaaacg
cbiX_for	agat*actagt*gaaacgggaggacattatatg
cbiX_rev	acaa*cccggg*cattttagctcgcccgacc
fnr_for	t*actagt*aattttctctctgttcgaccatc
fnr_rev	Gtgaat*gcatgc*ttctttcttgcc
metH*_for	tcagtc*actagt*tcaggcggagtggcgctcg
metH*_rev	gaagtc*gagctc*ttagcgcccgtgctgaatacgtac
glmS_for	tcagtc*actagt*atggaaaaaaagactatagtgcttg
glmS_rev	gaagtc*gagctc*cctgtcattatgtcatcctc
rtpR_for	tcagtc*actagt*atgagtgaagaaatatctctctcg
rtpR_rev	gaagtc*gagctc*ttagttcgctgttcagctggc

Restriction sites are shown in *italic* letters.

into the SpeI-KpnI restriction sites of the multiple cloning site of pHBintE to construct pHBihemA_{KK} (Barg *et al*., 2005). The DNA region coding for the antisense RNA of *B. megaterium hemZ* was amplified using primers ashemZ_for and ashemZ_rev. For the construction of pWH1520ashemZ, the 141 bp SpeI/BamHI digested PCR product was cloned into the appropriately cut pWH1520 (Rygus and Hillen, 1991). For the generation of pWH1520sirA, primers sirA_for and sirA_rev were used to amplify the *B. megaterium* gene *sirA* from genomic DNA of *B. megaterium* strain DSM509 by PCR. The PCR product and pWH1520 were digested with SpeI and BamHI and ligated. The new plasmid was named pWH1520sirA. For the generation of pHBicbiX, first pHBintT and pHBintN were constructed. The tetracycline-resistance gene and its control region were amplified using primers intT_for and intT_rev with pMM1522 (Malten *et al*., 2006) as a template and cloned via HindIII and PstI into the previously cut pHBintE (Barg *et al*., 2005) generating pHBintT. Next, primers intN_for and intN_rev were used to amplify the neomycin-resistance gene from pWH1967K (Schmiedel *et al*., 1997). Cloning of the NcoI/HindIII digested PCR product into the appropriately cut pHBintT resulted in pHBintN. The *cbiX* gene was amplified using primers cbiX_for and cbiX_rev with genomic DNA from *B. megaterium* strain DSM509 as template, digested with SpeI and XmaI, and cloned into the prior cut pHBintN to get pHBicbiX. For pWH1520fnr, the *B. subtilis* gene *fnr* was PCR-amplified from the genome of *B. subtilis* strain 168 using primers fnr_for and fnr_rev. Cloning of the SpeI/SphI digested PCR product into the appropriately cut pWH1520 resulted in pWH1520fnr.

The *metH** gene was amplified using primers metH*_for and metH*_rev from genomic *E. coli* DNA, the *rtpR* gene using primers rtpR_for and rtpR_rev from genomic *L. delbrueckii* strain DSM20076 DNA and the *glmS** gene using primers glmS_for and glmS_rev from plasmid pOZ3 (Zelder *et al*., 1994) by PCR. Cloning of the SpeI/SacI cut PCR products into the previously cut pMM1522 resulted in pMM1522metH*, pMM1522rtpR and pMM1522glmS, respectively.

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 (pHBintN-derivatives) were added to cultures carrying the various plasmids. Chromosomal integration processes of plasmid DNA was performed as described before (Barg *et al*., 2005). Chromosomally integrated P_{xylA} 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 B_{12} 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 B_{12} 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 B_{12} content was measured using the RIDASCREENFAST Vitamin B_{12} ELISA test (r-biopharm; Germany) according to the manufacture's introduction. All vitamin B_{12} 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 ddH_2O 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 (Luminescence Spectrometer LS50B, PerkinElmer, Boston, MA) to detect coproporphyrin III and protoporphyrin IX. For detection of coproporphyrinogen

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III and protoporphyrinogen IX, both were completely oxidized to coproporphyrin III and protoporphyrin IX, respectively, with 30% H_2O_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).

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