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Title: Effect of amino acid availability on vitamin B_{12} production in *Lactobacillus reuteri*

Running title: Increased B_{12} production in *Lactobacillus reuteri*

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1 **Abstract**

2 Recent functional genomics and genome-scale modeling approaches indicated that B_{12}
3 production in *Lactobacillus reuteri* could be improved by medium optimization. Here we show
4 that a series of systematic single amino acid omissions could significantly modulate the
5 production of B_{12} from nearly undetectable levels (by isoleucine omission) to 20-fold higher than
6 previously reported through omission of cysteine. We analyzed, by cDNA microarray
7 experiments, the transcriptional response of *L. reuteri* to the medium lacking cysteine. These
8 results supported the observed high B_{12} production and provided new avenues for future
9 improvement of production of vitamin B_{12}.

10

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1 **Introduction**

2 Vitamin B_{12} analogues (generally termed B_{12}) form a diverse subgroup of the tetrapyrroles, and
3 consist of structurally complex molecules that contain a ring-contracted porphinoid with cobalt
4 chelated at the core (5, 10). Synthesis of B_{12} is restricted to a few clades of bacteria and archaea,
5 while vitamin B_{12} auxotrophies are widely spread in prokaryotes, protists and animals, including
6 humans (11).
7 Vitamin B_{12} is a relevant compound from an anthropocentric perspective. It is an essential
8 dietary compound (vitamin) with a recommended nutrient intake of 2.4 μg/day for healthy adults
9 (4) and its deficiency has been associated with several pathologies, namely different forms of
10 anemia and neurological dysfunction, amongst others (19). Additionally, it is also an industrially
11 relevant compound since it is an essential co-factor in reactions incorporated in biotechnological
12 processes, such as the production of 1,3-propanediol (2).
13 *Lactobacillus reuteri* is a heterofermentative lactic acid bacterium that colonizes the gastro-
14 intestinal tract of humans and other animals (23). It has been shown to contain a functionally
15 active B_{12} biosynthetic gene cluster that encodes all the enzymes required for the synthesis of
16 this important co-factor from 5-aminolevulinate (12, 14). Catalyzed by glycerol dehydratase (EC
17 4.2.1.30), a B_{12}-dependent reaction, *L. reuteri* is able to synthesize 3-hydroxypropanaldehyde
18 (reuterin) (20). This compound with broad-spectrum antimicrobial activity (1) can be further
19 reduced to 1,3-propanediol restoring NAD^+, if coupled to the oxidation of another carbon source.
20 We have recently studied the glucose and glycerol co-fermentation by *L. reuteri* using functional
21 genomics and genome-scale modeling techniques. This enabled us to broaden our view of the
22 physiological responses of *L. reuteri* to glycerol, evidencing strong implications in amino acid

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1 metabolism and B_{12} biosynthesis (12). Here, we have focused on these new targets and attempted
2 to engineer the production of B_{12} in *L. reuteri* using physiological approaches. This led to the
3 discovery that single amino acid omissions can significantly affect the production of B_{12} in *L.*
4 *reuteri*. Hence, we studied the genome-wide transcriptional response of *L. reuteri* to the omission
5 of cysteine in search of mechanistic insights and new leads for metabolic engineering.
6
7 **Materials and Methods**

8 *Strains, media and culture conditions*. We obtained *L. reuteri* JCM1112 (type-strain, human
9 isolate) from the Japanese Collection of Microorganisms (Riken, Japan). It was cultured at 37ªC
10 in an adapted version of chemically defined medium (CDM) from which vitamin B_{12} was
11 omitted (22). When appropriate, 0.5% of glycerol was added. Cells were cultivated in standard
12 non-stirred batch cultures enclosed under an air atmosphere, unless stated otherwise. When
13 mentioned, oxygen availability was restricted by cultivating *L. reuteri* in anaerobic jars filled
14 with either O_2-free N_2, or a mixture of 95% N_2 and 5% CO_2.

15 *Single omission growth experiments.* We investigated for *L. reuteri*, the effect on B_{12} production
16 and growth behavior, of omitting separately every single amino acid that enters in the
17 composition of CDM. Parallel experiments were performed in the presence and absence of
18 glycerol, omitting an amino acid per culture and using a culture without any omission as a
19 reference. Independent biological duplos were carried out as described. An overnight culture of
20 *L. reuteri* washed twice in 0.85 % NaCl (w/v) was used to inoculate all media combinations to an
21 initial optical density at 600 nm (OD_{600}) of 0.05. Cultures were propagated in batch for five
22 consecutive transfers in the corresponding media monitoring growth every 24 hours. After 48

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1 hours, if we could not measure at least two doublings (OD_{600} < 0.2), the culture would be
2 discarded and not used for further analysis. If the fourth transfer had an OD_{600} above 0.5 after 24
3 hours of incubation, it was used to inoculate 20 ml of the corresponding media. This last culture
4 was used to determine the maximum specific growth rate (μ_{max}) and the B_{12} content.
5 *Growth rate determination. L. reuteri* JCM1112 was cultivated in the several variations of CDM,
6 in 96 well microtiterplates (Greiner, Alphen a/d Rijn, The Netherlands). Different replicates were
7 positioned on the plate using a checkerboard format distribution (3). Plates were incubated at
8 37°C in a Genios microtiterplate reader (Tecan, Zurich, Switzerland) set to monitor growth by
9 measuring OD_{595} every 15 min. All measurements were independently performed at least twice
10 in at least 8 biological replicates. μ_{max} was determined in a high-throughput fashion by in-house
11 scripts that calculate μ for every five consecutive time points through out the growth curve and
12 return its maximum value per well.
13 *Coenzyme B_{12} determination.* B_{12} production by *L. reuteri* in the different CDM variations was
14 determined according to the Official Methods of Analysis of AOAC International, using the *L.*
15 *delbrueckii* subsp. *lactis* ATCC 7830 vitamin B_{12} assay (6). Cell extracts for B_{12} analysis were
16 prepared as described elsewhere (13).
17 *Fermentation conditions.* pH-controlled batch fermentations of *L. reuteri* in the presence or
18 absence of cysteine were carried out in an experimental set-up consisting of four vessels with a
19 reaction volume of 400 ml. Prior to inoculation, the media vessels were gassed with O_2-free N_2
20 (15 ml/min) for 1 hour. Temperature was kept constant at 37°C and pH was fixed to 5.8 by
21 titration with 5 M NaOH. Homogeneity was assured by continuously stirring throughout the
22 whole fermentation. An exponentially growing culture in the corresponding media was used to

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1 inoculate the fermentors to an initial OD_{600} of 0.05. Periodic measurements of OD_{600} were used
2 to monitor biomass formation. Samples for transcriptome analysis were harvested at mid-
3 logarithmic growth phase (OD_{600} 1), and in early-stationary phase, 15 minutes after exponential
4 growth ceased (Fig. 1). At this stage of the growth curve, we also sampled for B_{12} analysis
5 (*idem*).
6 *Microarray design.* Dedicated microarrays for *L. reuteri* JCM1112 were spotted in the high
7 density Agilent 44K platform (Agilent Technologies, Santa Clara, CA, USA), based on the draft
8 genome sequence of *L. reuteri* JCM1112 released by JGI (retrieved in 03/2006). We used the
9 custom probe design covering 1700 (out of 1900) predicted coding regions (~90% coverage) that
10 we had developed for the 11K format available at GEO (http://www.ncbi.nlm.nih.gov/geo) under
11 accession number GPL6856, and had it printed four fold per array. Oligos were designed to
12 probe the predicted coding regions, representing 82.9% with 24 probes (6 unique) or more, and
13 only 3.9% with 12 (3 unique) or less.
14 *RNA isolation.* Cells harvested from cultures in the presence or absence of cysteine were
15 sampled by rapid quenching using a cold methanol method (9). Extraction and purification of
16 total RNA was carried out as previously described (12). RNA concentration was measured on a
17 ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA). The integrity of the mRNA
18 species was confirmed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A
19 threshold for the 23S/16S RNA ratio of 1.6 was set for the samples to be considered satisfactory.
20 Only samples above were used for transcriptome analysis.
21 *cDNA synthesis and labeling.* For each sample, 20 µg of RNA were used for first strand cDNA
22 synthesis using Superscript III reverse transcriptase (Invitrogen, Breda, The Netherlands)

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1 according to the recommendations of the manufacture. All samples of newly synthesized cDNA
2 were purified and labeled with cyanine 3 and cyanine 5. Indirect labeling was executed resorting
3 to the CyScribe first-strand cDNA labeling kit (Amersham, United Kingdom) following
4 manufacturer's recommendations. cDNA concentration and labeling incorporation were verified
5 using the ND-1000 spectrophotometer (NanoDrop Technologies Inc., USA).
6 *Microarray hybridization.* We used 0.8 μg of labeled cDNA of each dye per hybridization. All
7 samples were hybridized with each label at least once to facilitate the recognition of possible dye
8 effects. A hybridization scheme (supplemental material) consisting of a loop design comprising
9 13 microarrays was implemented to scrutinize the transcriptome of mid-logarithmic (exp-) and
10 early-stationary (sta-) growth phase cells cultured in pH-controlled batch fermentations of CDM
11 from which cysteine was omitted, or not. The hybridizations were performed at 60°C for 17 h,
12 after which, the slides were washed according to the recommendations of the manufacturer.
13 Scanning took place immediately after drying the slides as described elsewhere (16).
14 *Scanning and microarray data analysis.* Slides were scanned with a ScanArray Express scanner
15 (Perkin-Elmer) set to maximum resolution (5 μm). ImaGene (BioDiscovery) version 5.6 was
16 used for image analysis, spot quantification and data extraction. After normalizing the
17 transcriptome data by local fitting of an M-A plot applying the loess algorithm (24) using the
18 Limma package (17) in R (http://www.r-project.org), we analyzed the transcriptome data as
19 described elsewhere (16). Statistical significance was tested from the deviation between
20 biological duplicates by implementation of the eBayes function included in Limma (cross-probe
21 variance estimation) and false discovery rate adjustment of the *p*-values (18). Two comparisons
22 were established to characterize the impact in *L. reuteri* of removing cysteine (i) during

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1 exponential growth phase (mid-logarithmic phase response), and (ii) during early stationary
2 growth phase (early-stationary phase response). Transcripts were considered for analysis if the *p*-
3 value was smaller than 0.05 and the absolute Log_2 (ratio) greater than 0.585. The transcriptome
4 data was visualized by projection on metabolic maps of the genome-scale model developed
5 previously for *L. reuteri* (12).
6 *Microarray accession numbers.* The microarray platform developed in this study is available at
7 the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) (GPL7505). The microarray
8 data obtained were deposited in the same repository (GSE13391).
9
10 **Results**
11 We have recently observed that cultivation of *L. reuteri* in a chemically defined medium (CDM)
12 with glycerol has profound implications on B_{12} production and amino acid metabolism (12).
13 Here, we followed these newly obtained leads and attempt to modulate the production of B_{12} by
14 changing the amino acid composition of CDM and describing the phenotypic changes observed
15 related to growth and to the production of this co-factor.
16 *Single amino acid omissions and growth of L. reuteri.* We found that eight amino acids could be
17 omitted from CDM without completely inhibiting the growth of *L. reuteri*. These affected
18 growth rate and final biomass formation to variable degrees (Table 1). The omission of Serine
19 (Ser) profoundly reduced the specific growth rate and final optical density both in the absence or
20 presence of glycerol. In CDM lacking isoleucine (Ile) the final biomass formation is much more
21 affected than the growth rate. Omission of the other amino acids, namely alanine (Ala), aspartate
22 (Asp), cysteine (Cys), glycine (Gly), lysine (Lys), or proline (Pro) only slightly affected final

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1 biomass formation. The growth rates measured in all single amino acid omissions increased with
2 the addition of glycerol, except for CDM lacking Gly, which was only mildly affected (6%
3 reduction).

4 *Single amino acid omissions and vitamin B_{12} production. L. reuteri* JCM1112 produces
5 approximately 20 $\mu g.L^{-1}.OD_{600}^{-1}$ of B_{12} in complete CDM without glycerol. If the latter is added,
6 B_{12} production in *L. reuteri* increases about five fold to 100 $\mu g.L^{-1}.OD_{600}^{-1}$. When B_{12} production
7 was compared between *L. reuteri* cultures grown in complete, as opposed to single amino acid
8 deficient-CDM, a great disparity of effects was observed. (Table 2). The omission of Cys is the
9 most remarkable since, even without the addition of glycerol, it leads to a 5-fold increase in B_{12}
10 production relative to complete CDM. Furthermore, the boost in B_{12} synthesis can also be further
11 incremented by the presence of glycerol, enabling the accumulation of stimulatory effects,
12 resulting in over 350 $\mu g.L^{-1}.OD_{600}^{-1}$.

13 The increase in B_{12} production due to the addition of glycerol, although to varying proportions,
14 was observed regardless of the amino acid omissions from the composition of CDM. When Ile is
15 lacking, the largest stimulatory effect of glycerol is registered (20-fold). However, this is caused
16 by the sharp reduction (greater than 10-fold) in B_{12} production to levels barely detectable when
17 glycerol is absent, rather then by an increase of B_{12} production in CDM with glycerol.

18 Besides the effects already mention, in the absence of glycerol, *L. reuteri* produced higher
19 amounts of B_{12} in CDM lacking Lys, Ala, Gly and Asp, whilst in its presence, only the omission
20 of Ala and Asp had a positive effect.

21 *Effect of oxygen on B_{12} production.* Cysteine is known as a potent reducing agent. Omission of
22 cysteine will lead to increased redox potential and oxygen availability in the growth medium.

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1 The concentration of oxygen has been reported to affect negatively the production of B_{12} in
2 *Propionibacteria* (7). For this reason, we decided to check whether varying oxygen availability
3 could alter the stimulatory effect on B_{12} production caused by the omission of Cys in *L. reuteri*.
4 This was done by cultivating in parallel *L. reuteri* under an air atmosphere conditions, and in an
5 anaerobic vessel containing either pure N_2 or a mixture of 95% N_2 with 5% CO_2, and checking
6 B_{12} production in all possible variations of CDM combining the presence and absence of glycerol
7 and cysteine (Fig. 2). In 100% N_2, the production of B_{12} was slightly increased by about 30%.
8 The only exception was the condition in which cysteine is absent and glycerol is present, for
9 which there is only a trend, but no significant increase (p-value > 0.05). Cultivation in the
10 presence of 95% N_2 with 5% CO_2, caused the B_{12} content to increase slightly more, this time
11 about 50%. The trend observed for the cultures that combine the absence of cysteine with the
12 presence of glycerol is even more pronounced. Under this last condition B_{12} production was
13 raised to 428 ± 40 $\mu g.L^{-1}.OD_{600}^{-1}$.

14 *Transcriptomics of L. reuteri in absence of cysteine*. To gain further insight on the stimulatory
15 effect of Cys in B_{12} production, we studied the genome-wide transcriptional response of *L.*
16 *reuteri* to cultivation in the absence of Cys, by developing and performing cDNA microarray
17 experiments. We determined the specific responses in mid-exponential and early-stationary
18 growth phases. The complete list of loci that were found to be differentially regulated is available
19 in Table S1 (supplemental material) and a small selection that is discussed in the text is present
20 in Table 3.

21 In mid-exponential phase, we found 140 genes differentially regulated, of which only 8 were
22 found to be down-, while 132 were up-regulated. Concerning genes differently regulated in the

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1 stationary growth phase, we found 294 genes differentially regulated, 125 down- and 169 up-
2 regulated. A total of 58 genes are shared between both data sets, while 82 were exclusively
3 differentially regulated in exponential phase and 236 are specific for stationary phase.
4 In order to facilitate the analysis of the transcriptome data, we examined the distribution of the
5 genes found to be differentially regulated according to the functional class of the predicted
6 encoded protein, using the COG classification system (21). The full distribution is available at
7 Table S2 (supplemental material) and a selection is presented here based on its relevance for the
8 topics here discussed (Fig. 3).
9 Not surprisingly, the COG category associated with amino acid transport and metabolism was
10 overrepresented in both mid-exponential and stationary phase. Specific attention was given to
11 genes involved in cysteine metabolism. There are 2 genes (*lreu_1553* and *lreu_1792*) annotated
12 in the chromosome of *L. reuteri* that are predicted to encode cysteine synthase (EC 2.5.1.47) and
13 both are up-regulated in the absence of cysteine. The same holds for the genes (*lreu_0293-0295*,
14 *lreu_0502*, *lreu_1791*) coding for enzymes involved in the metabolism of sulfur-containing
15 compounds, such as cysteine desulfurase (EC 2.8.1.7), selenocysteine lyase (EC 4.4.1.16) and
16 cystathionine beta- and gamma-lyases (respectively, EC 4.4.1.8 and EC 4.4.1.1), amongst others.
17 Genes predicted to encode enzymes related to arginine, aspartate and ornithine tended to be
18 down-regulated (*lreu_0425*, *lreu_0426* and *lreu_0445*). The latter have in common that they lead
19 to the synthesis of carbomyl phosphate, a precursor of pyrimidines.
20 Harmoniously, genes associated with the COG category that includes proteins assigned to
21 nucleotide transport and metabolism, are not only overrepresented, but also display a clear
22 tendency to be down-regulated. Indeed, a closer look clearly shows that a complete cluster

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1 starting from *lreu_0123* to *lreu_0127* is drastically down-regulated in the absence of cysteine
2 (M-value around -5).
3 Co-factors such as folate derivatives are important in the synthesis of DNA and RNA precursors.
4 The folate biosynthesis gene cluster (*lreu_0510* and *lreu_1279-1280*, along with the one for
5 riboflavin (*lreu_0878-880*) appear down-regulated when cysteine is omitted from CDM and
6 account for roughly half of the regulated genes assigned to coenzyme transport and metabolism
7 (Table S1). As expected the B_{12} biosynthesis gene cluster was up-regulated.
8 An overrepresentation was observed for genes encoding proteins assigned to COG category
9 related to posttranslational modification and chaperons. Again the split between up- and down-
10 regulated appears quite even, implying that the absence of cysteine will not affect
11 indiscriminately all chaperons. Amongst others, in the down-regulated group we find loci
12 predicted to encode the co-chaperonin complex GroES and GroEL (*lreu_353-354*), while in the
13 up-regulated we can find thioredoxin, thioredoxin reductase (EC 1.8.1.9) and glutaredoxin
14 (*lreu_539*, *lreu_0376* and *lreu_0324*, respectively).
15 One final category worth highlighting is the ion transport and metabolism proteins. Within this
16 category, most genes were down-regulated, which can be ascribed mainly to the down-regulation
17 of unspecific ABC transporters. Highly relevant is the finding that the up-regulated group
18 includes genes involved in the scavenging of cobalt from the environment (*lreu_1707-1709*).
19
20 **Discussion**
21 The average B_{12} content of an early-stationary culture of *L. reuteri* JCM1112 in CDM is
22 approximately 20 $\mu g.L^{-1}.OD_{600}^{-1}$. This amount is increased about five fold if CDM is

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1 supplemented with glycerol. In these conditions, *L. reuteri* uses glycerol to recycle NAD+ via
2 the conversion of glycerol to 3-hydroxypropanaldehyde, which is subsequently reduced to 1,3-
3 propanediol (2). The co-fermentation of glucose and glycerol by *L. reuteri* has recently been
4 reported to have a major effect on the amino acid metabolism (12). In this study, we have
5 determined the effect on growth behavior and B_{12} production of individually omitting the amino
6 acids present in CDM. This was carried out in parallel in the presence and absence of glycerol.
7 In this screening exercise, we have identified eight amino acids to be essential for *L. reuteri* to
8 grow at least two generations in the first two days after inoculation. The well-established positive
9 effect of glycerol on the growth rate and B_{12} production of *L. reuteri* cultivated in complete
10 CDM was observed regardless of the omission of the single amino acids with the exception of
11 glycine (Table 1). When glycine is omitted, the addition of glycerol to CDM still has a positive
12 effect on B_{12} production (Table 2), but the effect on μ_{max} is lost. An explanation for this might
13 reside in the fact that the consumption rate of glycine in the presence of glycerol is nearly twice
14 as fast as in its absence (12). This is indicative of an increase demand for glycine in the presence
15 of glycerol, which cannot be met when *L. reuteri* has to rely solely on *de novo* synthesis. This
16 hypothesis is in agreement with the sharp decrease in μ_{max} observed in the absence of glycine and
17 glycerol, both for *L. reuteri* and *L. plantarum* (22).
18 In the absence of glycerol, the single omission of several amino acids lead to important
19 increments in B_{12} production, for instance aspartate 40%, glycine 60%, alanine 200% and lysine
20 240%. Any of these findings *per se* could lead to great improvements in B_{12} production
21 processes, especially the omission of lysine since it has a very little impact on the growth
22 behavior of *L. reuteri*. However, they are completely overshadowed by the 500% increase in B_{12}

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1 production caused by the omission of cysteine alone. Combined with the stimulatory effect of
2 glycerol, a 17-fold increase in B_{12} production was achieved (Table 2).
3 We have attempted to gain mechanistic insights and new clues on how to further increase the
4 synthesis of B_{12} in *L. reuteri* by studying its genome-wide transcriptional response to the
5 omission of cysteine. Due to the properties mentioned previously of cysteine as a reducing agent,
6 we started by carrying out an exploratory experiment under controlled oxygen availability. When
7 cultured anaerobically, *L. reuteri* was the first organism reported to produce exclusively
8 pseudovitamin B_{12}, presenting a great advantage for the downstream processing of its
9 purification. Under tight anaerobic conditions, B_{12} production was increased in *L. reuteri* by little
10 more than 30%. This could be anticipated, since *L. reuteri* has been shown to encode the oxygen-
11 independent biosynthetic route towards B_{12} (14), and the initial step of the industrial production
12 of B_{12} using *Propionibacteria* is carried out under strict anaerobic conditions (7). However, the
13 increase caused by the absence of cysteine from the medium was not diminished under
14 anaerobiosis, implying that this amino acid does indeed have other effects in the metabolism of
15 *L. reuteri*. When we compare B_{12} production of cells grown under an anaerobic environment
16 versus air, combining the omission of cysteine with the addition of glycerol, it is clear that even
17 though the trend is still there, the increase is not as high as expected. This suggests that after
18 increasing 20-fold the production of B_{12} another bottleneck is encountered.
19 The transcriptome analysis of cells grown in the absence of cysteine in comparison to the ones
20 grown in complete CDM confirms that indeed in *L. reuteri* cysteine is made from serine via
21 cysteine synthase, and using sulfur groups derived from methionine. As mentioned, *L. reuteri*
22 contains two copies of cysteine synthase genes (*lreu_1553* and *lreu_1792*), which are up-

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1 regulated in the absence of cysteine. We noticed that while the increased expression of the copy
2 encoded by *lreu_1792* changes very little between the exponential and stationary data sets, its
3 paralog, *lreu_1553* seems to be preferred in the later stages of growth. All pathways from amino
4 acids to carbomyl phosphate appeared downregulated along with the operon that encodes the
5 machinery necessary to channel it to pyrimidine biosynthesis. This is most likely related to the
6 drop in growth rate that we have characterized when cysteine is omitted from CDM (~15%
7 reduction).

8 The presence of the B_{12}-biosynthesis gene cluster among the genes that are upregulated in the
9 absence of Cys was quite expected taking into account the phenotype that we have exposed here.
10 The fact that we cannot find the full length of the cluster back as up-regulated can be easily
11 explained by the large size and fragility of the transcribed mRNA species as previously noted
12 while performing Northern blotting analysis (14). Nonetheless, if we loosen the stringency of our
13 established thresholds, the whole cluster will prove to have a positive M-value, instead of just the
14 genes in its centre. Based on the transcriptome data alone, *de novo* production of riboflavin and
15 folate seem to be negatively affected by the omission of Cys. Since both of these vitamins are
16 actually present in CDM in relative excess and we have recently illustrated metabolic
17 engineering strategies to increase folate production in *L. reuteri* (15), we decided not to pursue
18 this any further.

19 The omission of Cys has implications on sulfur metabolism that extend beyond its own
20 biosynthesis. The ubiquity of the loci encoding thioredoxin and thioredoxin reductase (EC
21 1.8.1.9) in both data sets and glutaredoxin in the stationary phase set is illustrative of such. These
22 are known to reduce other proteins by cysteine thiol-disulfide exchange, acting as antioxidants.

1 Here, seems to reside interactions yet to clarify, which could lead to even higher B_{12} production.
2 Partially related is cobalt bioavailability. We observed the up-regulation of genes for proteins
3 involved in the uptake of cobalt, which is logical since this metal is positioned at the core of the
4 tetrapyrrole macrocycle of B_{12} (10). Adding more cobalt to CDM supplemented with glycerol
5 and without cysteine does not yield significant increase in B_{12} production (data not shown),
6 which was not unexpected since CDM contains an excess of cobalt. This indicates that the rate of
7 cobalt supply is a limiting factor that is compensated by increased expression of one or more
8 cobalt transporters. Bioavailability of cobalt could be the bottleneck that limited the engineering
9 of B_{12}-production to a 20-fold increase.

10 Production of vitamin B_{12} relies solely on microbial production, since chemical methods remain
11 economically unviable due to the technical complexity of the synthesis process (8).
12 *Pseudomonas denitrificans* and *Propionibacteria* subsp. are accountable for most of the
13 industrially produced vitamin B_{12}, with a productivity as high as 300 mg.L^{-1} (8). We have used
14 physiological approaches to modulate the production of B_{12} in *L. reuteri* JCM1112 from nearly
15 undetectable levels to 20-fold higher than previously reported (15). Although, the production of
16 B_{12} obtained in this study using *L. reuteri* falls short of the maximum reported by nearly two
17 orders of magnitude, *L. reuteri* possesses the GRAS status and is a suitable host for *in situ*
18 production. Furthermore, we have characterized the impact on growth behavior of all cultivation
19 conditions tested, and analyzed by cDNA microarray experiments the transcriptional response of
20 *L. reuteri* to the omission of cysteine. This has lead to new insights that could be used to improve
21 even further the production of vitamin B_{12}.
22

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4

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1 **Figure Legends**

2

3 Figure 1. Biomass formation and sampling scheme in pH-controlled batch fermentations of
4 CDM in the presence (solid line) or absence (dashed line) of cysteine. Abbreviations mid-exp
5 and early-sta stand for mid-exponential and early-stationary growth phase, respectively.

6

7 Figure 2. Vitamin B_{12} production by *L. reuteri* under different gas environments in variations of
8 CDM. -Cys, cysteine omitted; +Glyc, supplemented with glycerol.

9

10 Figure 3. Distribution of transcripts whose expression was affected by the absence of cysteine
11 during early-stationary growth phase throughout a selection of COGs (see supplemental material,
12 Table S2 for full distribution). Transcripts were considered to be differentially regulated for $M >$
13 $|0.585|$ and p-value < 0.05. Light and dark grey bars represent proportion of up-regulated
14 transcripts in the absence or presence of cysteine, respectively. Between brackets is the total
15 number of genes from the genome of *L. reuteri* included in the corresponding COG.

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1 Table 1. Growth properties of *L. reuteri* in single amino acid deficient CDM supplemented or not
2 with glycerol [a.]

Amino acid omission	Conc.[b.] (mM)	CDM		CDM + glycerol		Variation CDM to CDM + glycerol
		μ_{max} (h^{-1})	Final OD_{595}	μ_{max} (h^{-1})	Final OD_{595}	
None	n.a.	0.453 (100%)	1.21 (100%)	0.575 (100%)	1.29 (100%)	27%
Ala	2.69	0.368 (81%)	1.17 (97%)	0.519 (90%)	1.31 (102%)	41%
Asp	3.16	0.445 (98%)	1.20 (99%)	0.551 (96%)	1.31 (102%)	24%
Cys	0.83	0.387 (85%)	1.16 (96%)	0.512 (89%)	1.26 (98%)	32%
Gly	2.33	0.370 (82%)	1.17 (97%)	0.349 (61%)	1.21 (94%)	-6%
Ile	1.60	0.356 (79%)	0.41 (34%)	0.411 (72%)	0.46 (36%)	15%
Lys	2.68	0.433 (96%)	1.20 (99%)	0.540 (94%)	1.27 (98%)	25%
Pro	5.86	0.424 (94%)	1.19 (98%)	0.555 (97%)	1.28 (99%)	31%
Ser	3.24	0.106 (23%)	0.25 (21%)	0.131 (23%)	0.24 (19%)	24%

3 [a.] Values are expressed as average (% of same condition without any amino acid omission) of at least
4 eight biological replicates. Experiment was repeated twice with similar results.
5 [b.] Concentration of amino acid in CDM when present (n.a., not applicable).

1 Table 2. Vitamin B_{12} production by *L. reuteri* in single amino acid deficient CDM supplemented
2 or not with glycerol [a.]

Amino acid omission	CDM	CDM + glycerol	Fold change from CDM to CDMglycerol	Fold change relative to reference condition[b.]
None	21.2 ± 3.6 (100%)	105.0 ± 17.0 (100%)	5	5
Ala	43.2 ± 4.7 (204%)	143.3 ± 10.9 (136%)	3	7
Asp	29.0 ± 4.8 (137%)	146.8 ± 10.4 (140%)	5	7
Cys	103.2 ± 13.0 (488%)	351.8 ± 28.4 (335%)	3	17
Gly	33.0 ± 3.3 (156%)	113.9 ± 14.1 (108%)	3	5
Ile	2.1 ± 0.4 (10%)	42.4 ± 5.8 (40%)	20	2
Lys	50.5 ± 5.1 (239%)	114.0 ± 22.5 (109%)	2	5
Pro	22.0 ± 4.7 (104%)	72.6 ± 11.9 (69%)	3	3
Ser	16.1 ± 3.6 (76%)	76.0 ± 10.4 (72%)	5	4

3 [a.] Values are expressed as average ± SD (% of same condition without any amino acid omission) of at
4 least three technical replicates. Experiment was repeated twice with similar results.
5 [b.] Reference condition is CDM without glycerol added and without any amino acid omission.

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1 Table 3. Selection of differentially regulated genes mentioned in the text (see supplemental
2 material, Table S1 for full list)

Locus	Product	M_{exp}	M_{sta}
Amino acid transport and metabolism			
Lreu_0190	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		-0.70
Lreu_0293	Cystathionine beta-lyase (EC 4.4.1.8) / Cystathionine gamma-lyase (EC 4.4.1.1)	1.72	
Lreu_0294	Cystine transport system permease protein	1.94	3.23
Lreu_0295	Cystine transport ATP-binding protein	2.26	3.16
Lreu_0348	Aspartate aminotransferase (EC 2.6.1.1)		-0.87
Lreu_0377	Succinyl-diaminopimelate desuccinylase (EC 3.5.1.18)		-1.24
Lreu_0425	Ornithine carbamoyltransferase (EC 2.1.3.3)	-3.38	-0.69
Lreu_0426	Carbamate kinase (EC 2.7.2.2)	-2.27	
Lreu_0445	Arginine deiminase (EC 3.5.3.6)	-2.09	
Lreu_0502	Cysteine desulfurase (EC 2.8.1.7)/Selenocysteine lyase (EC 4.4.1.16)		1.16
Lreu_0610	Diaminopimelate epimerase (EC 5.1.1.7)		-1.07
Lreu_0611	Aspartokinase (EC 2.7.2.4)		-1.51
Lreu_0612	Diaminopimelate decarboxylase (EC 4.1.1.20)		-0.70
Lreu_0613	Tetrahydrodipicolinate N-acetyltransferase (EC 2.3.1.89)		-0.65
Lreu_1553	Cysteine synthase (EC 2.5.1.47)	1.27	2.06
Lreu_1791	Homoserine O-succinyltransferase (EC 2.3.1.46)	0.80	1.27
Lreu_1792	Cysteine synthase (EC 2.5.1.47)	0.96	1.16
Nucleotide transport and metabolism			
Lreu_0123	Aspartate carbamoyltransferase (EC 2.1.3.2)		-5.32
Lreu_0124	Dihydroorotase (EC 3.5.2.3)		-5.36
Lreu_0125	Dihydroorotate dehydrogenase, catalytic subunit (EC 1.3.3.1)		-5.09
Lreu_0126	Orotidine 5'-phosphate decarboxylase (EC 4.1.1.23)		-4.91
Lreu_0127	Orotate phosphoribosyltransferase (EC 2.4.2.10)		-4.82
Coenzyme transport and metabolism			
Lreu_0510	Folylpolyglutamate synthase (EC 6.3.2.17)/Dihydrofolate synthase (EC 6.3.2.12)		-2.33
Lreu_0878	Diaminohydroxyphosphoribosylaminopyrimidine deaminase (EC 3.5.4.26) / 5-amino-6-(5-phosphoribosylamino)uracil reductase		-0.92
Lreu_0879	Riboflavin synthase alpha chain (EC 2.5.1.9)		-0.81
Lreu_0880	GTP cyclohydrolase II (EC 3.5.4.25) / 3,4-dihydroxy-2-butanone-4-phosphate synthase (EC 4.1.2.-)		-0.75
Lreu_1279	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase (EC 2.7.6.3)		-0.63
Lreu_1280	Dihydroneopterin aldolase (EC 4.1.2.25)		-0.73
Lreu_1710	Precorrin-2 C20-methyltransferase (EC 2.1.1.130)		0.71
Lreu_1711	Sirohydrochlorin cobaltochelatase (EC 4.99.1.3)		0.72
Lreu_1712	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107) / Uroporphyrinogen-III synthase (EC 4.2.1.75)		0.60
Posttranslational modification, protein turnover, chaperones			
Lreu_0324	Glutaredoxin		0.71
Lreu_0353	10 kDa chaperonin GROES		-0.83
Lreu_0354	60 kDa chaperonin GROEL		-0.84
Lreu_0376	Thioredoxin reductase (EC 1.8.1.9)	1.11	0.67
Lreu_0539	Thioredoxin	1.45	0.96
Inorganic ion transport and metabolism			
Lreu_1707	Cobalt transport protein cbiQ		0.85
Lreu_1708	Cobalt transport protein cbiN		0.63
Lreu_1709	CbiM protein		0.69

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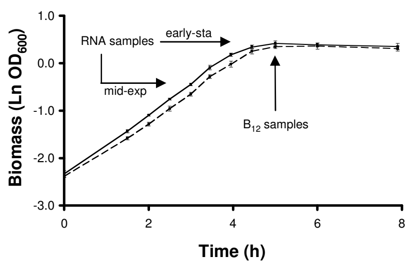

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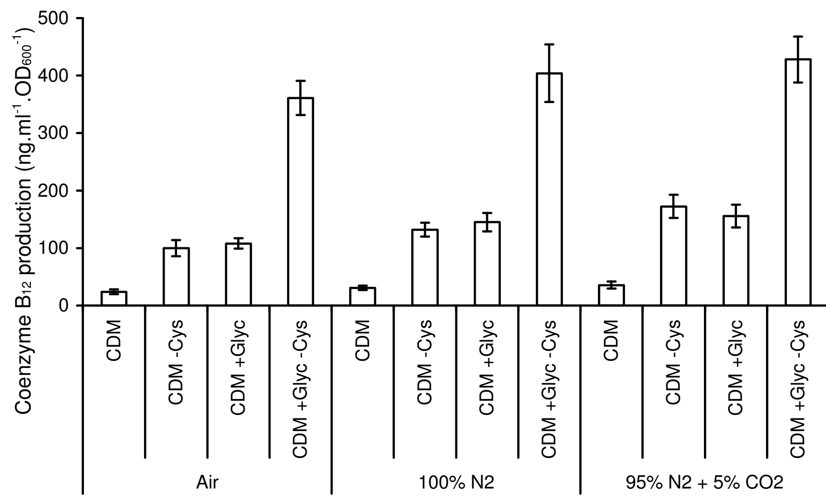

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