Available online at www.sciencedirect.com
SCIENCE DIRECT®
Metabolic Engineering 5 (2003) 49–55
METABOLIC ENGINEERING
http://www.elsevier.com/locate/ymben

# Reducing maintenance metabolism by metabolic engineering of respiration improves riboflavin production by *Bacillus subtilis*

Nicola Zamboni,[a] Nigel Mouncey,[b] Hans-Peter Hohmann,[b] and Uwe Sauer[a,*]

[a] *Institute of Biotechnology, Swiss Federal Institute of Technology Zürich, ETH Zürich, Honggerberg, CH-8093 Zürich, Switzerland*
[b] *Roche Vitamins AG, CH-4070 Basel, Switzerland*

Received 21 October 2002; accepted 30 January 2003

**Abstract**

We present redirection of electron flow to more efficient proton pumping branches within respiratory chains as a generally applicable metabolic engineering strategy, which tailors microbial metabolism to the specific requirements of high cell density processes by improving product and biomass yields. For the example of riboflavin production by *Bacillus subtilis*, we reduced the rate of maintenance metabolism by about 40% in a cytochrome *bd* oxidase knockout mutant. Since the putative Yth and the $caa_3$ oxidases were of minor importance, the most likely explanation for this improvement is translocation of two protons per transported electron via the remaining cytochrome $aa_3$ oxidase, instead of only one proton via the *bd* oxidase. The reduction of maintenance metabolism, in turn, significantly improved the yield of recombinant riboflavin and *B. subtilis* biomass in fed-batch cultures.


*Keywords:* Maintenance energy; Energy metabolism; Metabolic engineering; Respiration; Riboflavin

## 1. Introduction

Over the last decade, biotechnological production of many chemicals has become commercially competitive to preexisting chemical synthesis routes (Chotani et al., 2000). This development was largely driven by recombinant DNA technology, in what is now referred to as metabolic engineering (Bailey, 1991; Koffas et al., 1999; Nielsen, 2001). Once central metabolic and specific biosynthetic pathways are optimized for a given product, general traits of the production host govern biological, and thus, also commercial process performance. One important general trait is the rate of maintenance metabolism and, more specifically, the energetic investment for an organism to remain in a viable and active state without growth, which is quantified by the so-called non-growth associated maintenance energy coefficient (Pirt, 1965; Russell and Cook, 1995). This value is an intrinsic property of an organism and becomes a critical process variable in industrial fed-batch fermentations with slow growing cells (Stouthamer and van Verseveld, 1987). In fermentations for penicillin production, for example, about 70% of the carbon source is utilized for maintenance (Heijnen et al., 1979), thus detracting from the available resources for product or biomass formation.

Similarly, theoretical (Sauer and Bailey, 1999; Sauer et al., 1998) and experimental (Dauner et al., 2002; Sauer et al., 1996) evidence suggested that commercial production of riboflavin, vitamin B2, with recombinant *Bacillus subtilis* (Perkins et al., 1999) requires efficient energy generation and low maintenance metabolism. Since pathway engineering of riboflavin biosynthesis has attained a sophisticated level (Hümbelin et al., 1999) and precursor supply appears abundant (Dauner et al., 2001; Sauer et al., 1997), we attempt here to improve riboflavin production by increasing the efficiency of energy generation.

As in most other microbes (Richardson, 2000; Trumpower and Gennis, 1994), *B. subtilis* possesses a branched respiratory chain consisting of both quinol (encoded by the *cyd*, *qox* and *yth* operons) and cytochrome *c* (encoded by *ctaCDEF*) terminal oxidases (Fig. 1) (Calhoun et al., 1993; Neijssel and Teixeira de Mattos, 1994; Richardson, 2000). The use of a particular oxidase for energy generation is governed primarily by the environmental conditions such that energy coupling

*Corresponding author.
*E-mail address:* sauer@biotech.biol.ethz.ch (U. Sauer).

1096-7176/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S1096-7176(03)00007-7

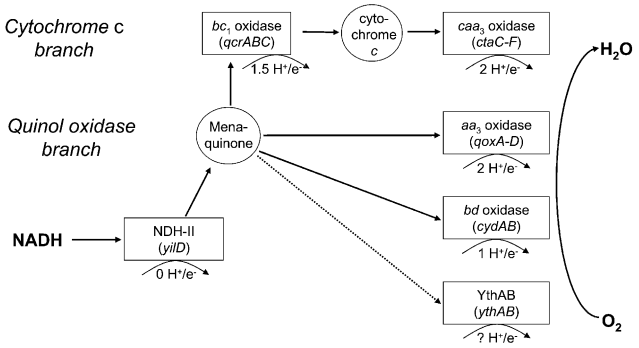


Fig. 1. Composition of the aerobic electron transport chain of *B. subtilis*. The dashed arrow indicates a putative pathway. The stoichiometry of energy coupling was estimated from the reported mechanisms of proton translocation (Trumpower and Gennis, 1994). NDH-II is the NADH:menaquinone reductase.

can be modulated to growth parameters e.g. availability of oxygen, carbon source, etc. Depending on the composition of the respiratory chain, between 1 and 3–4 protons may be translocated per transported electron (Sauer and Bailey, 1999; von Wachenfeldt and Hederstedt, 2002). The resulting proton gradient across the membrane, in turn, is then exploited for ATP generation via the $F_1F_0$ATP synthase. The industrially important Bacilli, in particular, appear to operate their respiratory chains well below the theoretical maximum (Christiansen and Nielsen, 2002; Sauer and Bailey, 1999; Sauer et al., 1996). The presented metabolic engineering strategy redirects the electron flow within the respiratory chain to a more efficient branch. By engineering cellular energetics and maintenance metabolism, we present a general strategy for improving otherwise optimized, industrial bioprocesses.

## 2. Materials and methods

### 2.1. Strains and molecular genetics

*B. subtilis* RB50 contains several chemically introduced purine and riboflavin analog-resistant mutations that deregulate the biosynthetic pathway (Perkins et al., 1991; 1999). In RB50::pRF69, the native, chromosomal *rib* operon is replaced by a copy of the constitutively expressed, recombinant *B. subtilis rib* operon, pRF69, that contains also a chloramphenicol resistance marker (Perkins et al., 1991; 1999). To increase production in fed-batch experiments with constant feed profiles, the recombinant *rib* operon was amplified by chloramphenicol selection (Dauner and Sauer, 2001). The resulting strain RB50::$[pRF69]_n$ contains multiple chromosomal copies of the recombinant *rib* operon.

The *qox*, *cyd*, and *yth* knockout mutations were initially constructed in *B. subtilis* 1012 by partial replacement of the relevant genes on the chromosome with antibiotic resistance cassettes. To construct a *qox* deletion–insertion mutation, a 2.2-kb DNA fragment containing the *qoxAB* genes, was amplified by PCR using *B. subtilis* wild-type strain 1012 genomic DNA as a template and primers QoxA+1 (5′-GAGAGGATCC-GAAAGGACCTGTAGC-3′) and QoxB-1 (5′-GAGA-GAATTCTTTCGTTCAGCTTGTGG-3′). The PCR product was purified using the QIAquick PCR purification kit (Qiagen Corp.) and was cloned directly into pGEM T-Easy vector, resulting in plasmid pSM7. Plasmid pSM7 was digested with *Bcl*I to remove a 1-kb fragment encompassing the very 3′ end of *qoxA* and the 5′ half of *qoxB*. The 1.2-kb spectinomycin-resistance cassette from plasmid pDG1726 (Guérout-Fleury et al., 1995) was amplified with PCR using primers Spc+1 (5′-GCGCTGATCATCGATTTTCGTTCGTG-3′) and Spc-1 (5′-GAGATGATCACCAATTAGAATG-3′). After purification and *Bcl*I digestion, the spectinomycin-resistance cassette was cloned into *Bcl*I-digested pSM7 to give plasmid pQox-Spec. Plasmid pQox-Spec was linearised and transformed into competent *B. subtilis* 1012 cells. Transformants were selected on TBAB plates containing spectinomycin to a final concentration of 100 μg/ml. To construct a *cydBC* deletion–insertion mutation, a 3.4-kb DNA fragment was amplified with PCR from the wild-type strain 1012 using primers CydA+1 (5′-GAGAGGATCCGATGT CTATCGGGC-3′) and CydC-1 (5′-GCGCGGATCCG TCAGGAAGG-3′). The purified PCR product was ligated into the pGEM-T Easy vector, resulting in plasmid pNMR20. Plasmid pNMR20 was digested with *Bcl*I removing 1.4-kb encompassing most of *cydB* and the 5′ half of *cydC*. The 1.2-kb neomycin-resistance cassette from plasmid pBEST501 (Itaya et al., 1989) was amplified using primers pBESTBcl+1 (5′-GAGATGAT-CAGCTTGGGCAGCAGGTCG-3′) and pBESTBcl-1 (5′-GAGATGATCATTCAAAATGGTATGCG-3′) using PCR, purified, digested with *Bcl*I, and cloned into *Bcl*I-digested pNMR20 to give plasmid pNMR21. pNMR21 was linearised with *Pst*I and transformed into *B. subtilis* wild-type strain 1012 and selected on TBAB plates containing neomycin to a final concentration of 5 μg/ml. To construct a *yth* deletion–insertion mutation, a 1.3-kb DNA fragment was amplified with PCR from the wild-type strain 1012 using primers YthA+1 (5′-TTGGCCAGAAGCCTTTTTGGCACG-3′) and YthA-12 (5′-GCGGAGCCATTACGACTCCGCTG-3′). The purified PCR product was ligated into the pGEM-T Easy vector, resulting in plasmid pNZ3. Plasmid pNZ3 was digested with *Bcl*I removing 648-bp in the middle of *ythA*. The 1.6-kb erythromycin-resistance cassette from plasmid pDG646 (Guérout-Fleury et al., 1995) was digested with *Bcl*I, and cloned into *Bcl*I-digested pNZ3 to give plasmid pNZ4. *Sca*I-linearised plasmid was transformed into *B. subtilis* wild-type strain 1012 and

Table 1
Constructed respiratory mutants of *B. subtilis* RB50::pRF69

| Strain | Relevant genotype |
|---|---|
| RB50::pRF69 | *spo0A rib::pRF69(cam)* |
| *cyd* | *spo0A rib::pRF69(cam) cydBC::neo* |
| *qox* | *spo0A rib::pRF69(cam) qoxAB::spc* |
| *yth* | *spo0A rib::pRF69(cam) ythAB::ery* |
| *cyd qox* | *spo0A rib::pRF69(cam) cydBC::neo qoxAB::spc* |
| *cyd yth* | *spo0A rib::pRF69(cam) cydBC::neo ythAB::ery* |

selected on TBAB plates with 0.5 μg/ml erythromycin. Correct integrations of the construct in the 1012 chromosome by double crossover were controlled by Southern hybridisation and PCR analysis. *B. subtilis* RB50::pRF69 mutants were obtained by bacteriophage PBS1 transduction from the constructed 1012 strains (Harwood and Cutting, 1990) (Table 1), and correct insertions were verified by PCR analysis.

### 2.2. Growth conditions and media

Strains for recombinant DNA experiments were grown in Luria–Bertani broth (LB). TBAB (Difco, Chicago, USA) was used for solid cultures on plate. Neomycin, spectinomycin, erythromycin, and/or chloramphenicol were added when needed to final concentrations of 5, 100, 0.5, and 20 mg/L, respectively. Seed cultures for shake flasks, chemostat, and fed-batch cultures were inoculated from frozen stocks and grown in M9 minimal medium (Harwood and Cutting, 1990) in the presence of antibiotics. All further cultivations were done without antibiotics. Batch cultures for physiological analyses were grown in shake flasks containing M9 minimal medium with 5 g/L glucose. Glucose-limited chemostat cultures were grown in minimal medium (Sauer et al., 1996) with 3.6 g/L glucose in a 1.5 L bioreactor (Bioengineering, Wald, Switzerland) at 37°C. Fully aerobic conditions with dissolved oxygen levels above 40% were ensured by a constant air flow of 1 L/min and an agitation speed of at least 1000 rpm.The continuous mode was initiated either slightly before or immediately after glucose depletion in batch culture.

Glucose-limited fed-batch cultures were grown in a 2 L bioreactor (Adaptive Biosystems, London, England). Quantitative physiological characterization was performed at 37°C in minimal medium containing in the batch 8 g/L glucose, 1.36 g/L $NH_4Cl$, 1.72 g/L $(NH_4)_2SO_4$, 0.05 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.4 g/L $KH_2PO_4$, 0.1 g/L NaCl, 0.16 g/L $MgSO_4 \cdot 7H_2O$, 19 mg/L $CaCl_2 \cdot 2H_2O$, 4 mg/L $FeSO_4 \cdot 7H_2O$, and 1.03 ml/L of trace salts with 0.9 g $MnCl_2 \cdot 4H_2O$, 525 mg $ZnCl_2$, 27 mg $CuCl_2 \cdot 2H_2O$, 40 mg $CoCl_2 \cdot 6H_2O$, 40 mg $Na_2MoO_4 \cdot 2H_2O$, and 100 mg $AlCl_3 \cdot 6H_2O$ per liter of stock solution. In the feed medium, the same components were added to 37.5-fold higher concentrations, resulting in a solution containing 300 g/L glucose. The feed was initiated at a rate of 1.55 ml/L(culture)/h and then increased exponentially at a rate of 0.05 $h^{-1}$ throughout the fermentation using a computer-controlled Ismatec MPC Standard pump and the Labworldsoft software (Ismatec SA, Glattbrugg, Switzerland).

For industrial process conditions, a partly complex medium and a constant feeding profile were used at a temperature of 39°C. The batch medium contained 0.75 g/L Na · glutamate, 4.71 g/L $KH_2PO_4$, 4.71 $K_2HPO_4$, 4.11 g/L $Na_2HPO_4 \cdot 2H_2O$, 0.23 g/L $NH_4Cl$, 1.41 g/L $(NH_4)_2SO_4$, 11.77 g/L yeast extract (Difco), 27.3 g/L glucose · $H_2O$, 1 g/L $MgSO_4 \cdot 7H_2O$, 62.5 mg/L $CaCl_2 \cdot 2H_2O$, 14.6 mg/L $MnSO_4 \cdot H_2O$, 4 mg/L $CoCl_2 \cdot 6H_2O$, 0.3 mg/L $Na_2MoO_4 \cdot 2H_2O$, 1 mg/L $AlCl_3 \cdot 6H_2O$, 0.8 mg/L $CuCl_2 \cdot 2H_2O$, 4 mg/L $ZnSO_4 \cdot 7H_2O$, and 40 mg/L $FeSO_4 \cdot 7H_2O$. The feed solution containing 655.2 g/L glucose · $H_2O$ was supplied at an initial rate of 13.3 ml/L/h for 2 h and at 14.7 ml/L/h for the remainder of the cultivation. Stirring speed at 1500 rpm and air flow between 3 and 5 L/min ensured dissolved oxygen levels above 15% throughout the cultivations.

### 2.3. Analytical techniques and determination of physiological parameters

Concentrations of carbon dioxide and oxygen in the reactor feed and effluent gas were determined with a mass spectrometer (Prima 600, Fisons Instruments). Concentrations of cellular dry weight (CDW) was determined from a least eight parallel 10-ml cell suspensions, which were harvested by centrifugation, washed with distilled water, and dried at 110°C for 24 h to a constant weight. For riboflavin measurements, culture samples were diluted with 0.2 M NaOH to the linear range of the spectrophotometer and the $A_{440}$ was immediately measured. Glucose, riboflavin, organic acids, acetoin, and diacetyl in the culture supernatant were determined as described previously (Dauner et al., 2002; Sauer et al., 1996).

In batch culture, the exponential growth phase was identified by log-linear regression analysis of biomass concentration versus time, with growth rate ($\mu$) as the regression coefficient. Physiological parameters in chemostat cultures were determined in steady state, defined as at least 5 volume changes after adjusting to a new dilution rate ($D$) and stable optical density as well as oxygen uptake and carbon production rate for minimally 1 volume change. Under such steady-state conditions, consumption and production rates of substrates ($S$) and products ($P$) were determined from their concentration difference in the feed medium (or air) and the effluent broth (or gas). Specific consumption or production rates ($q_s$ or $q_p$) were obtained from the relationship $q_s$ (or $q_p$) $= \Delta S$ (or $P$) (D/$X$), where $X$ is the steady-state biomass concentration. Using the

relationship

$$q_{glc} = \mu(Y^{max})^{-1} + m_{glc}, \quad (1)$$

where $Y^{max}$ is the maximum molar growth yield and $m_{glc}$ is the maintenance coefficient (Pirt, 1965; Russell and Cook, 1995), the maintenance requirements in terms of glucose were calculated by linear regression analysis of $q_{glc}$ at different $D$ as the intercept with the Y-axis. These $q_{glc}$ values were corrected for the glucose required for riboflavin formation.

## 3. Results and discussion

### 3.1. Construction of respiration mutants

A series of mutants with altered respiratory chains was constructed by transduction of the *qox*, *cyd*, and/or *yth* operons (Fig. 1) that were partially substituted by inserted antibiotic resistance markers into the riboflavin-producing *B. subtilis* strain RB50::pRF69 (Dauner et al., 2002; Perkins et al., 1991; Perkins et al., 1999) (Table 1). The phenotypic characteristics were quantitatively assessed in batch cultures grown in glucose-containing minimal medium. Growth of the *cyd* and *yth* single and double mutants was indistinguishable from their parent with a $\mu_{max}$ of about $0.45\,h^{-1}$, while the *qox* mutant grew significantly slower at a $\mu_{max}$ of $0.36\,h^{-1}$ as was described earlier (Santana et al., 1992), and the *cyd qox* double mutant did not grow at all on minimal medium. In contrast to the *B. subtilis* type strain 168 (Winstedt and von Wachenfeldt, 2000), the RB50::pRF69 *cyd qox* double mutant grew under aerobic conditions on complex medium. However, growth was extremely weak, since the double mutant attained $OD_{600}$ values of maximally 0.1, while all others grew to values exceeding 2. These results indicate that neither the YthAB nor the cytochrome *c* oxidase branch contribute significantly to electron flow during exponential growth in batch culture.

Largely in agreement with results obtained with the type strain 168 (Winstedt and von Wachenfeldt, 2000), our experiments reveal that the $aa_3$ and *bd* quinol oxidases are the predominant constituents of the *B. subtilis* respiratory chain under the conditions investigated. While their relative contributions to electron flow are unknown, either oxidase is fully capable of supporting growth alone. Consequently, RB50::pRF69 *cyd* is expected to rely primarily on the $aa_3$ oxidase branch, and thus to exhibit a higher energetic coupling efficiency than the parent (Fig. 1). Since RB50::pRF69 *cyd* is defect in maturation of cytochrome *bd* (*cydC* deletion), we can exclude the function of other, potentially unrecognized cytochrome *bd* oxidases (Winstedt and von Wachenfeldt, 2000).

### 3.2. Maintenance metabolism

To assess the impact of potentially increased energetic coupling efficiency on growth, RB50::pRF69 and its *cyd* mutant were grown in glucose-limited chemostat cultures over a large range of dilution rates. To avoid potential occurrence of mutants with reduced riboflavin productivity (Sauer et al., 1996), we inoculated cultures newly for each steady state. While the steady state biomass concentration of the control exhibited the typical hyperbolic correlation with $D$, the *cyd* mutant biomass concentration decreased above a critical $D$ of about $0.075\,h^{-1}$ (Fig. 2), revealing reduced biomass yields that were accompanied by enhanced overflow metabolism to acetate (data not shown). Below the critical $D$, however, the mutant used the available glucose more efficiently and grew to higher concentrations than the control. This improved biomass yield at low $D$s indicates that the *bd* oxidase catalyzes a significant fraction of the electron flow in slow-growing *B. subtilis* under carbon limitation, and that the $aa_3$ oxidase can readily substitute this function. Since both strains had comparable specific oxygen consumption and carbon dioxide production rates above the critical $D$ (Fig. 3), the apparent growth defect and the associated overflow metabolism of the *cyd* mutant at high $D$s is seemingly not related to insufficient $aa_3$ oxidase activity. A possible explanation for this behavior is reduced oxygen scavenging by the $aa_3$ oxidase at high rates of respiration. Consistently, the *B. subtilis* $aa_3$ oxidase affinity for oxygen is approximately 100-fold lower than that of the *bd* oxidase (Garcia-Horsman et al., 1991; Rice and Hempfling, 1978). Thus, electron flow through the $aa_3$ oxidase alone may not be sufficient to eliminate excess reducing equivalents, hence leading to deleterious effects on cell growth. Alternatively, increased ATP

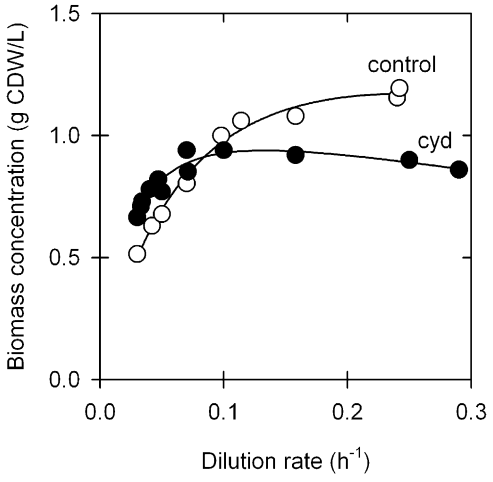


Fig. 2. Effect of dilution rate in glucose-limited chemostat culture on the steady-state biomass concentration of RB50::pRF69 (control) and its *cyd* mutant.

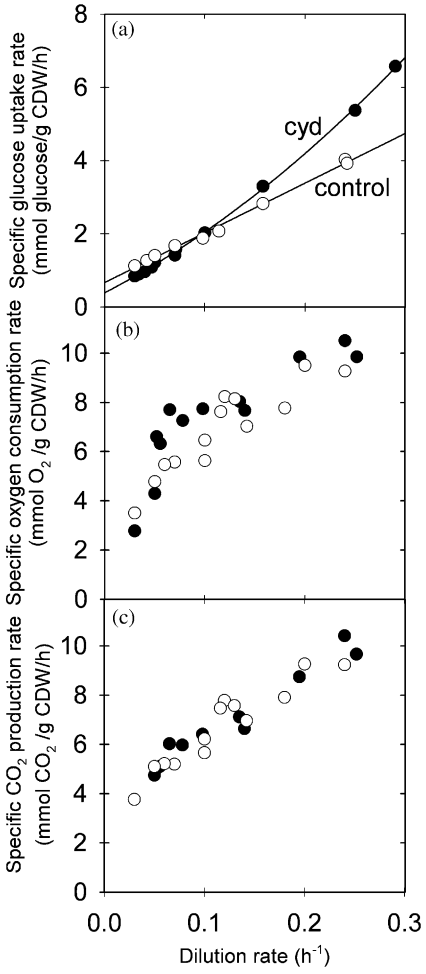


Fig. 3. Effect of dilution rate in glucose-limited chemostat culture on the specific rate of glucose consumption (a), oxygen uptake (b), and carbon dioxide production (c) of RB50::pRF69 (control) and its *cyd* mutant. Lines in (a) represent the best fit from linear regression or a second-order polynomial fit for the control and *cyd* mutant, respectively.

generation may trigger overflow metabolism in the *cyd* mutant at higher $D$ values.

To quantify the rate of maintenance metabolism, we plotted the specific glucose uptake rate ($q_{glc}$) as a function of $D$ (Fig. 3a). The control strain exhibited a linear correlation, which, when extrapolated to a $D$ of zero, yielded a non-growth associated maintenance energy coefficient of 0.67 mmol glucose/g CDW/h. This value compares favorably with the previously reported value of 0.65 mmol/g/h for a similar riboflavin-producing *B. subtilis* (Sauer et al., 1996). In contrast, the *cyd* mutant showed a non-linear correlation with significantly lower $q_{glc}$ values below the critical $D$ than in the control. The corresponding maintenance coefficient of 0.39 mmol/g/h is 40% lower than that of the control. A very likely explanation for this reduced maintenance demand is the increased coupling efficiency of respiratory energy generation by electron flow redirection from the *bd* to the $aa_3$ oxidase.

Previous work on *Escherichia coli* respiratory mutants showed that the rate of respiration depends on the coupling efficiency of the aerobic respiratory chain, since an improvement of the coupling efficiency leads to lower specific oxygen consumption rates and vice versa (Calhoun et al., 1993). In contrast to *E. coli*, the rate of respiration in *B. subtilis* appears not to be driven directly by the energy demand because we found similar specific oxygen consumption rates.

### 3.3. Riboflavin production in exponential fed-batch culture

Based on the hypothesis that efficient supply of ATP is critical for production of riboflavin (Dauner et al., 2002; Sauer and Bailey, 1999), the apparently reduced energetic expenditures for maintenance metabolism should increase riboflavin formation in RB50::pRF69 *cyd*. Hence, both strains were grown in minimal medium fed-batch cultures with an exponentially increasing feed rate of 0.05 $h^{-1}$ profile that ensures a growth-limiting but constant specific glucose supply per g cells. Since both strains grew exponentially with an experimentally determined rate of 0.045 $h^{-1}$ (Fig. 4) their biomass yields were similar. However, the riboflavin yield on glucose of the *cyd* mutant was 0.032 g g, compared to 0.028 g g for the control at around 24 h. Consequently, a 30% higher riboflavin titer was accumulated by the *cyd* mutant compared to the control after 48 h. Thus, the fed-batch culture conditions apparently favored riboflavin production rather than higher biomass yields, as was seen in the previous chemostat experiments.

### 3.4. Riboflavin production in industrial fed-batch culture

To achieve commercially relevant riboflavin titers the recombinant *rib* operons of both strains were amplified, yielding RB50::$[pRF69]_n$ and RB50::$[pRF69]_n$ *cyd*. Both

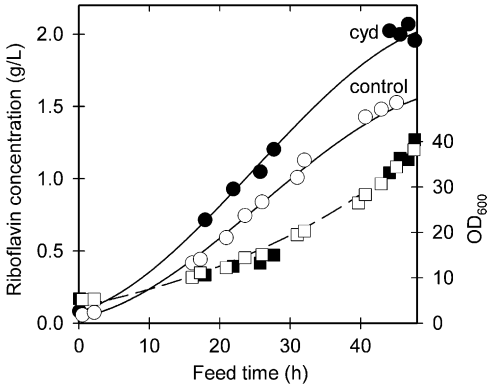


Fig. 4. Time profiles of riboflavin concentration (circles) and $OD_{600}$ (squares) of *B. subtilis* RB50::pRF69 (open symbols) and its *cyd* mutant (closed symbols) in glucose-limited fed-batch culture (minimal medium) with an exponentially increasing feed rate of 0.05 $h^{-1}$.

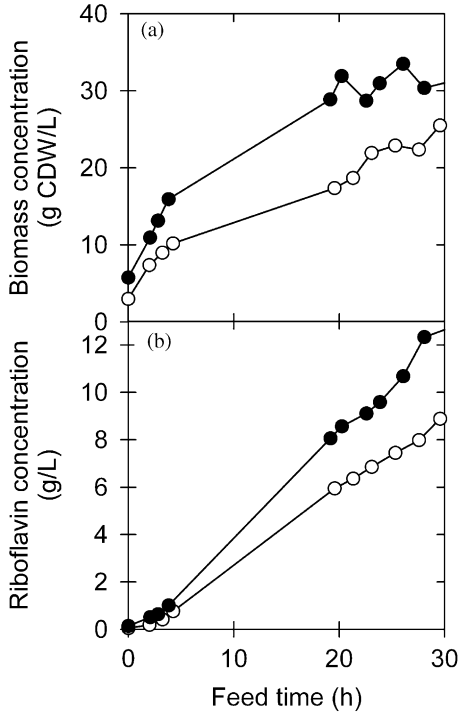


Fig. 5. Time profiles of biomass (a) and riboflavin concentration (b) of *B. subtilis* RB50::[pRF69]$_n$ (open symbols) and its *cyd* mutant (closed symbols) in glucose-limited fed-batch culture (complex medium) with a constant feeding profile. Representative examples of at least two independent cultivations with each strain are shown. The biomass concentration was calculated from $OD_{600}$ measurements, using 0.33 g CDW/L per unit $OD_{600}$ as conversion factor.

strains were then grown in complex medium fed-batch cultures using a feeding profile from the industrial process. After conclusion of the 6 h batch phase, feeding was initiated at a cell density of about 6 g CDW/L and previously accumulated metabolic by-products such as acetate and acetoin were co-metabolized with glucose and presumably complex medium components during the initial feeding period. During the initial feed phase, the RB50::[pRF69]$_n$ *cyd* mutant grew faster and accumulated significantly more riboflavin than the control, 12.3 and 8.9 g/L after 30 h, respectively (Fig. 5). Although after 20 h feeding both strains grew below the critical rate, at this point the *cyd* mutant biomass yield is only 0.05 g CDW/g glucose compared to 0.08 g CDW/g for the control. This is presumably a consequence of the higher concentration of accumulated biomass for the *cyd* mutant and thus reduced availability of glucose per cell at this time point. While the riboflavin yield decreased in both strains over time, the *cyd* mutant exhibited consistently higher yields than the control (0.041 and 0.032 g riboflavin/g glucose for the *cyd* mutant and the control after 24 h, respectively). Thus, increasing the efficiency of energy coupling not only reduces maintenance requirements, but also improves the overall yield of recombinant riboflavin production.

The presented results suggest that increasing respiratory energy generation is a general strategy to improve product yields in industrial bioprocesses with significant maintenance metabolism. Typical cases are high cell density fed-batch cultures, and one such example could be amino-acid production with *Corynebacterium glutamicum*, which, like *B. subtilis*, employs alternative electron transport chains of the *bd*- and *aa*$_3$-type with different coupling efficiencies (Niebisch and Bott, 2001).

## References

Bailey, J.E., 1991. Toward a science of metabolic engineering. Science 252, 1668–1675.

Calhoun, M.W., Oden, K.L., Gennis, R.B., de Mattos, M.J., Neijssel, O.M., 1993. Energetic efficiency of *Escherichia coli*: effects of mutations in components of the aerobic respiratory chain. J. Bacteriol. 175, 3020–3025.

Chotani, G., Dodge, T., Hsu, A., Kumar, M., LaDuca, R., Trimbur, D., Weyler, W., Sanford, K., 2000. The commercial production of chemicals using pathway engineering. Biochim. Biophys. Acta 1543, 434–455.

Christiansen, T., Nielsen, J., 2002. Growth energetics of an alkaline serine protease-producing strain of *Bacillus clausii* during continuous cultivation. Bioproc. Biosyst. Eng. 24, 329–339.

Dauner, M., Sauer, U., 2001. Stoichiometric growth model for riboflavin-producing *Bacillus subtilis*. Biotechnol. Bioeng. 76, 132–143.

Dauner, M., Storni, T., Sauer, U., 2001. *Bacillus subtilis* metabolism and energetics in carbon-limited and excess-carbon chemostat culture. J. Bacteriol. 183, 7308–7317.

Dauner, M., Sonderegger, M., Hochuli, M., Szyperski, T., Wüthrich, K., Hohmann, H.-P., Sauer, U., Bailey, J.E., 2002. Intracellular carbon fluxes in riboflavin-producing *Bacillus subtilis* during growth on two-carbon substrate mixtures. Appl. Environ. Microbiol. 68, 1760–1771.

Garcia-Horsman, J.A., Barquera, B., Escamilla, J.E., 1991. Two different *aa*$_3$-type cytochromes can be purified from the bacterium *Bacillus cereus*. Eur. J. Biochem. 199, 761–768.

Guérout-Fleury, A.M., Shazand, K., Frandsen, N., Stragier, P., 1995. Antibiotic-resistance casettes for *Bacillus subtilis*. Gene 167, 335–336.

Harwood, C.R., Cutting, S.M., 1990. Molecular Biological Methods for Bacillus. John Wiley & Sons Ltd, Chichester, England.

Heijnen, J.J., Roels, J.A., Stouthamer, A.H., 1979. Application of balancing methods in modeling the penicillin fermentation. Biotechnol. Bioeng. 21, 2175–2201.

Hümbelin, M., Griesser, V., Keller, T., Schurter, W., Haiker, M., Hohmann, H.-P., Ritz, H., Richter, G., Bacher, A., van Loon, A.P.G.M., 1999. GTP cyclohydrolase II and 3,4-dihydroxy-2-butanone 4-phosphate synthase are rate-limiting enzymes in riboflavin synthesis of an industrial *Bacillus subtilis* strain used for riboflavin production. J. Ind. Microbiol. Biotechnol. 22, 1–7.

Itaya, M., Kondo, K., Tanaka, T., 1989. A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. 17, 4410.

Koffas, M., Roberge, C., Lee, K., Stephanopoulos, G., 1999. Metabolic engineering. Annu. Rev. Biomed. Eng. 1, 535–557.

Neijssel, O.M., Teixeira de Mattos, M.J., 1994. The energetics of bacterial growth: a reassessment. Mol. Microbiol. 13, 172–182.

Niebisch, A., Bott, M., 2001. Molecular analysis of the cytochrome $bc_1$–$aa_3$ branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome $c_1$. Arch. Microbiol. 175, 282–1294.

Nielsen, J., 2001. Metabolic engineering. Appl. Microbiol. Biotechnol. 55, 263–283.

Perkins, J.B., Pero, J.G., Sloma, A., 1991. Riboflavin overproducing strains of bacteria. European patent 0 405 370 A1.

Perkins, J.B., Sloma, A., Hermann, T., Theriault, K., Zachgo, E., Erdenberger, T., Hannett, N., Chatterjee, N.P., Williams II, V., Rufo Jr, G.A., Hatch, R., Pero, J., 1999. Genetic engineering of *Bacillus subtilis* for the commercial production of riboflavin. J. Ind. Microbiol. Biotech. 22, 8–18.

Pirt, S.J., 1965. The maintenance energy of bacteria in growing cultures. Proc. R. Soc. London Ser. B, 224–231.

Rice, C.W., Hempfling, W.P., 1978. Oxygen-limited continuous culture and respiratory energy conservation in *Escherichia coli*. J. Bacteriol. 134, 115–124.

Richardson, D.J., 2000. Bacterial respiration: a flexible process for a changing environment. Microbiology 146, 551–571.

Russell, J.B., Cook, G.M., 1995. Energetics of bacterial growth: balance of anabolic and catabolic reactions. Microbiol. Rev. 59, 48–62.

Santana, M., Kunst, F., Hullo, M.F., Rapoport, G., Danchin, A., Glaser, P., 1992. Molecular cloning, sequencing, and physiological characterization of the *qox* operon from *Bacillus subtilis* encoding the $aa_3$-600 quinol oxidase. J. Biol. Chem. 267, 10225–10231.

Sauer, U., Bailey, J.E., 1999. Estimation of P-to-O ratio in *Bacillus subtilis* and its influence on maximum riboflavin yield. Biotechnol. Bioeng. 64, 750–754.

Sauer, U., Hatzimanikatis, V., Hohmann, H.-P., Manneberg, M., van Loon, A.P., Bailey, J.E., 1996. Physiology and metabolic fluxes of wild-type and riboflavin-producing *Bacillus subtilis*. Appl. Environ. Microbiol. 62, 3687–3696.

Sauer, U., Hatzimanikatis, V., Bailey, J.E., Hochuli, M., Szyperski, T., Wuthrich, K., 1997. Metabolic fluxes in riboflavin-producing *Bacillus subtilis*. Nat. Biotechnol. 15, 448–452.

Sauer, U., Cameron, D.C., Bailey, J.E., 1998. Metabolic capacity of *Bacillus subtilis* for the production of purine nucleosides, riboflavin, and folic acid. Biotechnol. Bioeng. 59, 227–238.

Stouthamer, A.H., van Verseveld, H.W., 1987. Microbial energetics should be considered in manipulating metabolism for biotechnological purposes. Trends Biotechnol. 5, 149–155.

Trumpower, B.L., Gennis, R.B., 1994. Energy transduction by cytochrome complexes in mitochondrial and bacterial respiration: the enzymology of coupling electron transfer reactions to transmembrane proton translocation. Annu. Rev. Biochem. 63, 675–716.

von Wachenfeldt, C., Hederstedt, L., 2002. Respiratory cytochromes, other heme proteins, and heme biosynthesis. In: Sonenshein, A.L., Hoch, J.A., Losick, R. (Eds.), *Bacillus subtilis* and Its Close Relatives. ASM Press, Washington, DC, pp. 163–179.

Winstedt, L., von Wachenfeldt, C., 2000. Terminal oxidases of *Bacillus subtilis* strain 168: one quinol oxidase, cytochrome $aa_3$ or cytochrome *bd*, is required for aerobic growth. J. Bacteriol. 182, 6557–6564.