COMMUNICATION TO THE EDITOR

Estimation of P-to-O Ratio in *Bacillus subtilis* and Its Influence on Maximum Riboflavin Yield

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Abstract: Simultaneous growth and riboflavin overproduction were investigated using a previously developed stoichiometric model of *Bacillus subtilis* metabolism. A fit of model predictions to experimental data was used to obtain estimates of fundamental energetic parameters of *B. subtilis.* Although multiple solutions describe the experimental data, evidence for a P-to-O ratio of about 1⅓ mole of ATP produced per atom of oxygen consumed in oxidative phosphorylation was provided by genomic analysis of electron transport components, because no homologue of the proton-translocating NADH dehydrogenase I was found in the *B. subtilis* genome database. These results allow us to devise a rational metabolic engineering strategy to improve riboflavin production. The potential influence of increased energy coupling in oxidative phosphorylation on riboflavin yield is discussed. Higher coupling is most significant under carbon-limiting conditions in slow-growing cells, that is, in fed-batch processes of industrial interest. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **64**: 750–754, 1999.

Keywords: *Bacillus subtilis;* bioenergetics; metabolic engineering; P/O; riboflavin

INTRODUCTION

For most industrial microorganisms, biochemical knowledge is sufficient to describe the stoichiometry of their metabolic systems and thus allows estimation of maximum theoretical yields of products or biomass which, in turn, can indicate metabolic bottlenecks. On the other hand, evaluating the influence of cellular energetics on yields is less straightforward, because the energetic parameters of an organism are not readily obtained from experimental data. A major difficulty resides in the variability of stoichiometric ratios in respiratory metabolism (e.g., H^+/e^- in proton translocation and H^+/ATP in ATP synthesis). However, microbial energetics are important for practical reasons because they influence the limits to which the yield of a number of biotechnological products, including riboflavin (vitamin B_2), can be improved (Sauer et al., 1998; Stouthamer and van Verseveld, 1987).

Production of riboflavin can be achieved by various microorganisms among which *Bacillus subtilis* currently has the highest commercial potential (van Loon et al., 1996). High level production strains of *B. subtilis* are obtained by a combination of two strategies (Perkins et al., 1991). First, feedback inhibition is eliminated by traditional mutagenesis and selection procedures in the purine nucleotide biosynthesis pathway that supplies the guanosine triphosphate precursor for riboflavin biosynthesis. Additionally, selection for roseoflavin resistance, a riboflavin analog, leads to deregulating mutations in *ribC*, which possibly encodes a flavinkinase and/or FAD synthase (catalyzing the conversion of riboflavin to the coenzymes FMN and FAD) (Coquard et al., 1997). Second, riboflavin biosynthesis proteins are overexpressed in deregulated strains from the strong, constitutive phage promoter *spol-15* in various artificial operon constructs that are integrated in the chromosome.

Although high riboflavin titers exceeding 15 g/L are achieved after about 50 h of fed-batch fermentation, the overall riboflavin yields on glucose are relatively low, in the range of 0.05 g/g (Perkins et al., 1991). In a previous study, we showed that cellular energetics may be a limiting factor for riboflavin production by investigating the general metabolic capacity of nongrowing *B. subtilis* using a flux balance approach (Sauer et al., 1998). Here we present a detailed case study on energetics of *B. subtilis* and theoretical riboflavin production in growing cells, including the potential impact of improved ATP generation in oxidative phosphorylation.

METHODS

Simulations

A previously constructed stoichiometric model of *B. subtilis* metabolism was used as published (Sauer et al., 1998).

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Briefly, mass balances on cellular metabolites provide a set of linear equations relating intracellular and external fluxes. To obtain optimal flux distributions and maximum yields, a constrained optimization problem was formulated in which production or consumption of a particular metabolite (or biomass) is maximized/minimized subject to

$$\mathbf{S} \cdot \mathbf{v} \geq \mathbf{b} \text{ or } \mathbf{S} \cdot \mathbf{v} \leq \mathbf{b} \quad (1)$$

where **S** is the stoichiometric matrix of the metabolic network, **v** is the vector of unknown reaction fluxes, and **b** is the production rate vector for all metabolites in the network. Cell growth was incorporated in terms of metabolite requirements on the basis of a cell composition analysis. The solution was computed by linear optimization with a single objective using MATLAB (The MathWorks, Inc., Natick, MA). The objective functions examined were maximizing growth and riboflavin production, the latter at an assumed glucose uptake rate derived from experimental data. In all simulations, the gluconeogenic phosphoenolpyruvate carboxykinase was considered to be inactive.

RESULTS AND DISCUSSION

Estimation of Bioenergetic Efficiency

An overall mass balance of intracellular ATP from the consumption site can be written in the form

$$q_{ATP} = m_{ATP} + \mu/Y_{ATP}^{max} \quad (2)$$

where q_{ATP} is the ATP production rate (mmol g^{-1} h^{-1}), m_{ATP} is the ATP consumption rate for non-growth-associated maintenance (mmol g^{-1} h^{-1}), μ is the specific growth rate (h^{-1}), and Y_{ATP}^{max} is the theoretical growth yield on ATP (g $mmol^{-1}$) (Stouthamer and Bettenhausen, 1973). In respiratory metabolism, q_{ATP} can be written from the production site as

$$q_{ATP} = P_S + P/O \cdot 2P_R \quad (3)$$

where P_S is the amount of ATP synthesized via substrate phosphorylation (mmol g^{-1} h^{-1}), P_R is the amount of reducing equivalents that deliver electrons to the respiratory chain (mmol g^{-1} h^{-1}), and the P-to-O ratio (P/O) is the amount ATP produced per atom oxygen consumed. While it is clear that P/O and Y_{ATP}^{max} cannot be estimated independently, combinations of both parameters can be found that are consistent with the experimental data.

Previously, we have determined the specific glucose demand for non-growth-associated maintenance (m_{glc}) of wild-type *B. subtilis* as 0.45 mmol of glucose g^{-1} h^{-1} by the standard approach of measuring the specific glucose uptake rate (q_{glc}) as a function of dilution rate in a glucose-limited chemostat (Sauer et al., 1996) according to the following equation (Russell and Cook, 1995):

$$m_{glc} = q_{glc} - \mu/Y_{glc}^{max} \quad (4)$$

To relate m_{glc} to m_{ATP}, we used the known yields of NADH and ATP in glycolysis and TCA cycle (12 and 4, respectively) according to

$$m_{ATP} = m_{glc}(12 \cdot P/O + 4) \quad (5)$$

To attribute values to the two remaining parameters of the combined Eqs. (2) and (3), P/O and Y_{ATP}^{max}, we fitted model predictions to the above described experimental data. At each simulated μ, P_S and P_R were calculated by the stoichiometric model. In the parameter fitting procedure either P/O or Y_{ATP}^{max} were assumed to be constant and the other value was estimated from the best fit to the experimental data. Figure 1 shows the example of a fitting procedure in which P/O was assumed to be 1. The best fit was obtained with a Y_{ATP}^{max} of 11.9 g mol^{-1}. However, there are multiple solutions that describe the experimental data, all of which can be expressed as the combinations of P/O and Y_{ATP}^{max} given in Fig. 2.

Electron Transport Chain Composition in *B. subtilis*

Compared with the Gram-negative bacteria, little is known about energy transduction in the respiratory metabolism of *B. subtilis* (Trumpower and Gennis, 1994). To see if there is any biological rationale for a lower P/O in *B. subtilis* than the maximum value of 2–3 assumed for *Escherichia coli* (Gottschalk, 1986), we performed homology searches for respiratory chain components in the recently completed genome database of *B. subtilis* (Kunst et al., 1997) using the gapped Blast algorithm (Altschul et al., 1997). The major electron donor to the respiratory chain is NADH, and *E. coli*

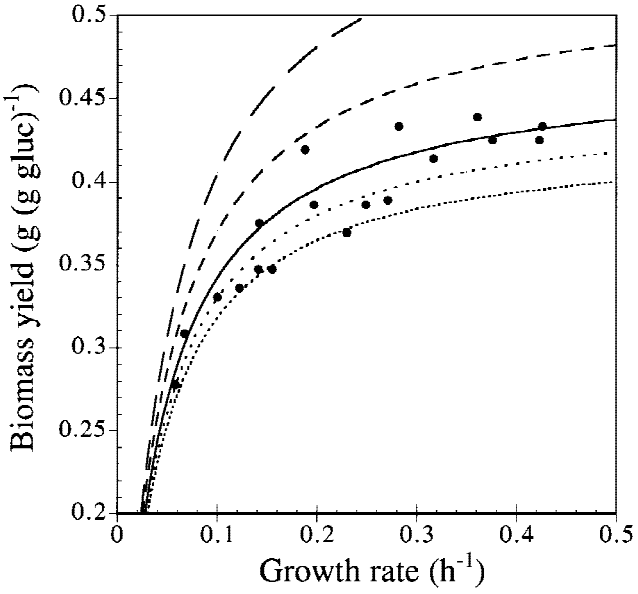

Figure 1. Experimental and simulated *B. subtilis* biomass yields in glucose-limited chemostat culture as a function of specific growth rate. Simulations were performed with a P/O of 1, a growth-independent maintenance demand of 7.2 mmol ATP g^{-1} h^{-1} and Y_{ATP}^{max} of 26.3, 16.7, 11.9, 11.1, or 10 (top to bottom) g (cells) mol^{-1}. Experimental data (filled circles) are from Sauer et al. (1996).

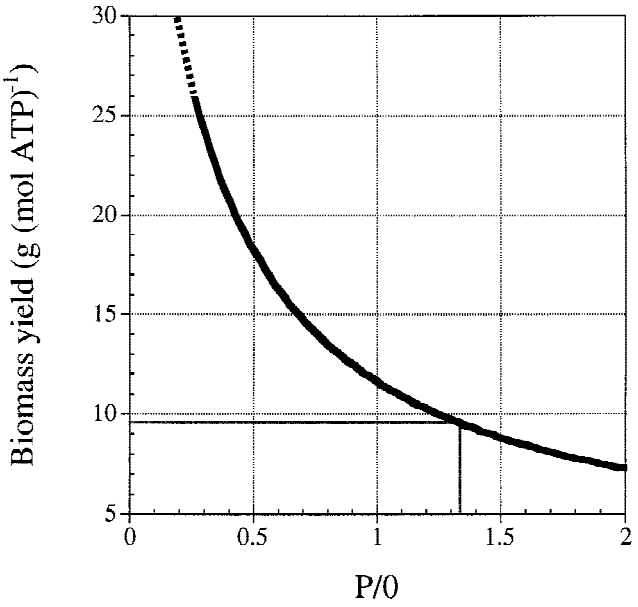

Figure 2. Feasible combinations of P/O and Y_{ATP}^{max} that describe experimentally determined growth yields of *B. subtilis* in the stoichiometric model. The dashed line at P/O below 0.26 indicates solutions with ATP requirements that are less then that calculated from composition data, i.e. 38 mmol ATP g^{-1} (equivalent to a Y_{ATP}^{max} of 26.3 g mol^{-1}). The gray lines indicate the most likely P/O and Y_{ATP}^{max} of glucose-grown *B. subtilis.*

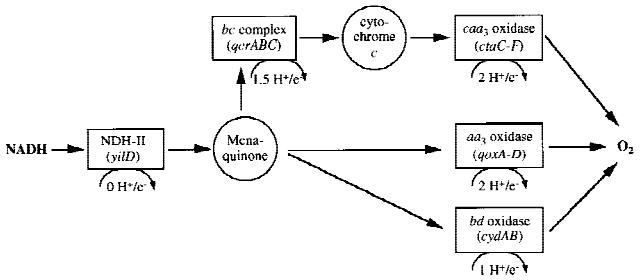

Figure 3. Composition of the aerobic electron transport chain in *B. subtilis.* Components are compiled from experimental data (Liu and Taber, 1998; Santana et al., 1992; Winstedt et al., 1998; Yu et al., 1995) and genomic database information (Kunst et al., 1997). The stoichiometry of energy coupling was estimated from the reported mechanisms of proton translocation (Trumpower and Gennis, 1994).

contains two NADH:ubiquinone oxidoreductases (Unden and Bongaerts, 1997). One enzyme, the multisubunit NADH dehydrogenase I (NDH-I) (encoded by *nuoA-N*), couples electron transfer to proton translocation and thus transduces redox energy into a proton gradient. The alternative enzyme, the monomeric NDH-II (encoded by the *ndh* gene) is not proton translocating (Calhoun et al., 1993). Homology searches revealed that the *B. subtilis* genome contains an analogue (YilD) of the *E. coli* NDH-II. On the other hand, no analogues were found in the *B. subtilis* genome for the 14 NuoA-N polypeptides of *E. coli*, with the exception of the as yet uncharacterized *B. subtilis* NdhF protein, which shows some sequence similarity to one of the two membrane-spanning subunits (29% identity to NuoL in a 273-amino acid stretch). This similarity, however, is significantly lower than that of other NDH-I subunits from unrelated bacteria to each other. Thus, *B. subtilis* apparently lacks the NDH-I coupling site (Fig. 3) unless another, not yet identified, energy-coupling NDH exists. This, however, is unlikely since the 14-subunit *E. coli* NDH-I is considered to be the minimal form of a proton-translocating NDH (Unden and Bongaerts, 1997).

Multiple terminal oxidases, which catalyze the transfer of electrons to oxygen with different energy-coupling efficiencies, exist in bacteria with branched electron transport chains such as *E. coli* and *B. subtilis.* In *E. coli*, the H^+/e^- stoichiometry of the terminal *bo* and *bd* oxidases was determined to be 2 and 1, respectively (Unden and Bongaerts, 1997), and at least 1.5 for NDH-I (Bogachev, et al., 1996). If we assume that three protons are required by ATP synthase to drive phosphorylation of one ATP (Gottschalk, 1986), the maximum P/O in *E. coli* would be 2⅓, with NDH-I contributing one P/O unit. *B. subtilis* is known to contain three terminal oxidases, two of which accept electrons from menaquinone and one from cytochrome *c* (Fig. 3). The main electron flow in glucose-grown *B. subtilis* is via the aa_3 oxidase (Santana et al., 1992) while the *bd* oxidase appears to be relevant mainly at low oxygen tension (Winstedt et al., 1998). The cytochrome *c* branch, which also contains the energy-coupling menaquinone:cytochrome *c* oxidoreductase (*bc* complex), is subject to catabolite repression and therefore undetectable in glucose-grown *B. subtilis*, but is expressed during growth on nonfermentable carbon sources (Liu and Taber, 1998; Yu et al., 1995). Thus, in glucose-grown *B. subtilis*, the maximum P/O appears to be 1⅓, a value that compares very well with the overall respiratory H^+/e^- stoichiometry of 1.9 determined from oxygen pulse experiments with exponentially growing *B. subtilis* (Lauraeus and Wikström, 1993). According to Fig. 2, this P/O correlates to a Y_{ATP}^{max} of about 9.5 g mol^{-1}.

In principle, it should thus be possible to engineer *B. subtilis* strains with improved energy prosperity that exhibit a higher P/O. One strategy would be functional expression of *nuoA-N* from *E. coli* combined with concomitant disruption of *yilD.* The normal electron acceptor of NDH-II in *B. subtilis* is menaquinone ($E_0' = -74$ mV) rather than the ubiquinone ($E_0' = +113$ mV) used by *E. coli.* Despite the lower redox difference between NADH ($E_0' = -320$ mV) and menaquinone, NDH-I of *E. coli* was shown to function with this electron acceptor (Tran et al., 1997), although lower turnover rates of NDH-I may limit glucose catabolism due to NADH accumulation. However, this consequence is unlikely to be relevant in slow-growing cells under glucose limitation such as those in typical fed-batch cultivations. A second strategy would involve simultaneous relief of the cytochrome *c* branch from catabolite repression and disruption of the aa_3 and *bd* oxidases. This strategy is particularly promising as riboflavin-producing *B. subtilis* usually carries the *spoOA* mutation (Perkins et al., 1991; Sauer et al., 1996), which represses expression of at least the *bc* complex (Yu et al., 1995). A conceptually simpler strategy would involve deleting the *bd* oxidases. Potentially adverse kinetic

consequences of this deletion for operation at very low partial oxygen pressure can be avoided in the fermentation process by sufficient aeration. Proof of this concept has already been provided in a quantitative study on respiratory chain mutants of *E. coli,* which exhibited higher yields on oxygen when NDH-II and the *bd* oxidase were eliminated (Calhoun et al., 1993).

Maximum Riboflavin Yields in Slow-Growing *B. subtilis*

Previously we determined the theoretical riboflavin yield in nongrowing *B. subtilis* by using riboflavin formation as the objective function in a stoichiometric model of cellular metabolism (Sauer et al., 1998). However, in practice the product yield will also be affected by simultaneous biomass generation. Here we focus on the industrially relevant process of riboflavin production in slow-growing cells. To assess maximum yields, riboflavin production was optimized from a given specific glucose consumption rate at stepwise-reduced biomass formation (μ) (Fig. 4). The experimentally determined value of wild-type *B. subtilis* at this q_{glc} in glucose-limited chemostat was taken as the initial μ (Sauer et al., 1996). Specifically, glucose consumption rates of 1.67 and 2.89 mmol g^{-1} h^{-1} were used, which correspond to those of *B. subtilis* growing at μ of 0.1 and 0.2 h^{-1}. Reduced growth rates at constant q_{glc} and m_{ATP} simulate an increased drain of glucose for product formation. The extent of this drain, and thus the maximum riboflavin yield at a given P/O, depends in practice on the biosynthetic and the catabolic capacity of the actual strain used. The energetic parameters, P/O, m_{ATP}, and Y_{ATP}^{max} estimated above for wild-type *B. subtilis* were used in these calculations. Additionally, P/O values greater than 1 are shown in Fig. 4 to simulate the consequences of metabolic engineering to increase the stoichiometric efficiency of oxidative phosphorylation, as described in the previous section. At each assumed P/O, the two other parameters were kept constant.

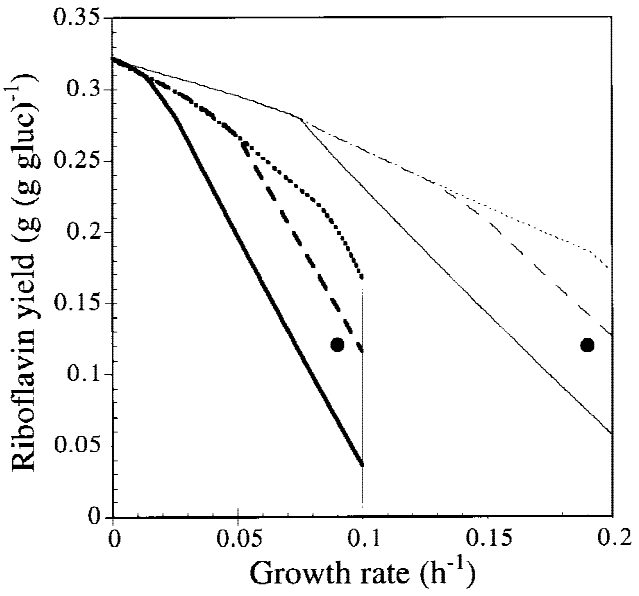

Figure 4. Influence of μ and P/O on maximum riboflavin yields on glucose at constant glucose consumption rates. The horizontal dimension (with decreasing μ) indicates increased riboflavin formation relative to biomass formation, hence is a function of the actual strain used. The vertical dimension represents the efficiency of energy generation for a given strain. Thin lines indicate a q_{glc} of 2.89 mmol g^{-1} h^{-1} and bold lines indicate a q_{glc} of 1.67 mmol g^{-1} h^{-1}, as were determined for glucose-limited chemostat cultures of *B. subtilis* growing at a dilution rate of 0.2 and 0.1 h^{-1}, respectively (Sauer et al., 1996). Thus, the lines start at the growth rate that corresponds to the assumed q_{glc} (indicated by the vertical line for q_{glc} of 1.67) and merge at the theoretical maximum yield of riboflavin in non-growing cells. P/O values of 1, 1½, and 2 are represented by solid, dashed, and dotted lines, respectively. The filled circles illustrate the maximum riboflavin yield for the *B. subtilis* strain PRF93, as estimated from the chemostat data (Sauer et al., 1996) assuming a P/O of 1⅓.

At the energetic efficiency estimated for *B. subtilis,* there is very little capacity for riboflavin production, unless glucose consumption is increased above (or μ is decreased below) the level displayed by the wild-type under carbon-limited conditions (Fig. 4). The discontinuity in slope indicates a shift from energetic to stoichiometric limitation of product formation. At μ close to 0, the riboflavin yield approaches the previously determined maximum value of 0.322 g g^{-1} (0.154 mol mol^{-1}) and is then limited by the network stoichiometry (Sauer et al., 1998).

Clearly, if q_{glc} could be increased to high values at low growth rates, the efficiency of energy transduction is less relevant for riboflavin production. Although nongrowing bacteria consume carbon, the rate of this consumption is lower than in growing cells and cannot be maintained for extended periods (Weikert et al., 1998), and thus they are generally not sufficient for commercial processes. However, small increases in glucose consumption for product formation can more reasonably be expected from engineered production strains. For example, the riboflavin-producing *B. subtilis* strain PRF93 has 11.4 and 5.9% higher q_{glc} than the wild-type at μ of 0.1 and 0.2 h^{-1}, respectively (Sauer et al., 1996, 1997). In the plot shown in Fig. 4, a reduction in μ from 0.10 to 0.09 h^{-1} and from 0.20 to 0.19 h^{-1} at P/O of 1 corresponds to the above increases in q_{glc}. For strains that correspond to this region, where riboflavin formation is limited by the availability of energy, the maintenance requirements become a critical factor. Any increase of this parameter inevitably decreases the achievable riboflavin yield.

CONCLUSIONS

The results indicate that glucose-limited wild-type *B. subtilis* exhibits a P/O of about 1⅓, a m_{ATP} of about 9 mmol ATP g^{-1} h^{-1}, and a Y_{ATP}^{max} around 9.5 g mol^{-1} (equivalent to an ATP demand of 105 mmol per gram of newly formed biomass). In principle, it is possible to improve the energetic prosperity, and potential benefits of engineered oxidative phosphorylation on riboflavin production are particularly important when small (realistic) changes in substrate consumption for product formation are considered in growing cells. Generally, the yield could be increased by 20% if P/O

could be increased from 1⅓ to just 1½, although improved ATP production in an engineered strain is also likely to increase biomass formation. Thus, we conclude that production strains with even small increases in P/O could achieve much higher riboflavin yields than do normal *B. subtilis.* This yield improvement cannot be achieved by a further overexpression of riboflavin biosynthesis components, because in bacteria the rate of carbon consumption is correlated with the growth rate, and thus glucose consumption for riboflavin formation is coupled to biomass formation.

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