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Screening of *Bacillus subtilis* transposon mutants with altered riboflavin production

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ABSTRACT

To identify novel targets for metabolic engineering of riboflavin production, we generated about 10,000 random, transposon-tagged mutants of an industrial, riboflavin-producing strain of *Bacillus subtilis*. Process-relevant screening conditions were established by developing a 96-deep-well plate method with raffinose as the carbon source, which mimics, to some extent, carbon limitation in fed batch cultures. Screening in raffinose and complex LB medium identified more efficiently riboflavin overproducing and underproducing mutants, respectively. As expected for a "loss of function" analysis, most identified mutants were underproducers. Insertion mutants in two genes with yet unknown function, however, were found to attain significantly improved riboflavin titers and yields. These genes and possibly further ones that are related to them are promising candidates for metabolic engineering. While causal links to riboflavin production were not obvious for most underproducers, we demonstrated for the gluconeogenic glyceraldehyde-3-phosphate dehydrogenase GapB how a novel, non-obvious metabolic engineering strategy can be derived from such underproduction mutations. Specifically, we improved riboflavin production on various substrates significantly by deregulating expression of the gluconeogenic genes *gap*B and *pck*A through knockout of their genetic repressor CcpN. This improvement was also verified under the more process-relevant conditions of a glucose-limited fed-batch culture.

1. Introduction

Biotechnological production of many chemicals has become commercially competitive to traditional chemical production, but the overall fraction of biotechnologically produced compounds is still relatively small (Hatti-Kaul et al., 2007). A prominent example of a biological process that has superseded chemical synthesis is production of vitamin B_2 (riboflavin). While several cell factories such as the fungi *Ashbya gossypii*, *Eremothecium ashbyii*, *Candida flareri* and the bacterium *Bacillus subtilis* were successfully engineered for riboflavin production (Stahmann et al., 2000), the current commercial processes rely on *A. gossypii* and *B. subtilis*. In the case of *B. subtilis*, production strains were developed by the combination of random chemical mutagenesis, screening and rational metabolic engineering (Perkins et al., 1991, 1999). Initially, the purine pathway to the riboflavin precursor GTP was deregulated by selecting mutants with resistance to purine-analogs. Subsequently, strong constitutive expression of riboflavin biosynthesis genes was achieved by integrating a recombinant *rib* operon into the chromosome. To further increase biosynthetic enzyme dosage, chloramphenicol selection was used to amplify the recombinant *rib* operons on the chromosome.

On the basis of this combined rational and classical strain development (Perkins et al., 1991, 1999), commercial riboflavin production with *B. subtilis* has been going on for almost a decade. During this last decade, many, if not all, obvious additional targets for metabolic engineering have been interrogated. Examples include the overexpression of key riboflavin biosynthesis genes (Humbelin et al., 1999), decoupling cell growth from production (Hohmann et al., 2003) and increasing precursor supply by redirecting carbon flow through the pentose phosphate pathway (Gershanovich et al., 2000; Zamboni et al., 2004). A main limiting factor for production was found to be the efficient supply of energy (Sauer and Bailey, 1999), and thus strategies were developed to improve ATP formation through cofeeding experiments (Dauner et al., 2002) or by deleting the less efficient branch of the respiratory chain (Zamboni et al., 2003). While some of these strategies have found their way into the production process,

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the achieved riboflavin yields fall still significantly short of the theoretical maximum yield (Sauer et al., 1998) and the improvements tend to become smaller.

We thus wondered whether the above targets were exclusive or at least the most relevant ones, or whether yet not understood properties beyond riboflavin biosynthesis and cellular energetics are relevant for riboflavin production. To identify such novel targets for metabolic engineering, we applied a random, transposon-tagged mutagenesis approach to the industrial riboflavin-producer *B. subtilis* RB50::pRF69 (Perkins et al., 1991). The underlying rationale is that once genes are known whose inactivation causes increased or decreased riboflavin production, novel metabolic engineering strategies can be devised, as has been demonstrated in earlier applications of transposon mutagenesis (Alper et al., 2005a, b; Ni et al., 2007). To improve the probability that process-relevant genes are identified, we developed a novel liquid screening system that was based on the slow metabolism of the tri-hexose saccharide raffinose, which in batch experiments mimics to some extent glucose limitation in industrial fed-batch processes.

2. Materials and methods

2.1. Strains and molecular genetics

B. subtilis RB50::pRF69 (*spo0A*, *purA60*, Az^r-11, Dc^r-15, MS^r-46, RoF^r-50) contains several chemically introduced purine and riboflavin analog-resistant mutations that deregulate the biosynthetic pathway to riboflavin (Perkins et al., 1991, 1999). The native *rib*-operon has further been replaced by a recombinant operon that is expressed from two strong constitutive promoters and contains a chloramphenicol resistance marker (Perkins et al., 1991, 1999).

For transposon mutagenesis, the mini-Tn*10* delivery vector pIC333 was introduced into strain *B. subtilis* RB50::pRF69. This vector contains a 2.4-kb mini-Tn*10* containing a ColE1 origin and a spectinomycin resistance gene, an erythromycin resistance gene, a thermosensitive origin of replication for Gram-positive hosts and a Tn*10* transposase gene (Steinmetz and Richter, 1994). Upon protoplast transformation (Harwood and Cutting, 1990), transformants were selected at 30 °C on DM3 plates (per liter: 135 g sodium succinate, 5 g casamino acids, 5 g yeast extract (YE), 6 g glucose, 20 mmol $MgCl_2$, 0.1 g bovine serum albumin, and 8 g agar) supplemented with 0.5 μg/mL erythromycin. Transformant colonies were used to inoculate 2 mL cultures of Luria-Bertani (LB) broth (per liter: 5 g YE, 10 g trypton, and 10 g NaCl) supplemented with 100 μg/mL spectinomycin. After 3 h of incubation at 30 °C, the temperature was raised to 37 °C and cells were grown for an additional 4 h. Aliquots were stored at −20 °C for further use. For screening purposes, a few microliters from the frozen mutant pool were plated on LB agar plates with 100 μg/mL spectinomycin and incubated at 37 °C.

2.2. Growth conditions and media

Screening for riboflavin over and underproducing Tn*10* transposon mutants was carried out in 2-mL-deep well microtiter plates with a culture volume of 1 mL (Duetz et al., 2000). Upon orbital shaking at 300 rpm at a shaking diameter of 5 cm, these microtiter plates attain oxygen mass transfer rates similar to baffled shake flasks. Picked mutant colonies were grown for 30–48 h at 37 °C under the above conditions in LB medium or M9 minimal medium (per liter: 5.64 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.246 g $MgSO_4 \cdot 7H_2O$, 0.014 g $CaCl_2$, at pH 7.4), where the latter was supplemented with 10 mL trace salts solution (per liter: 1.35 g $FeCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot H_2O$, 0.17 g $ZnCl_2$, 0.043 g $CuCl_2 \cdot 2H_2O$, 0.06 g $CoCl_2 \cdot 6H_2O$, 0.06 g $Na_2MoO_4 \cdot 2H_2O$) (Harwood and Cutting, 1990) and 5 g/L raffinose. Only in screening cultures, an additional 0.2 g/L YE was added to the minimal medium, and this medium is referred to as raffinose YE. Shake flask batch cultures were grown at 37 °C in 500-mL baffled flasks with 50 mL of M9 minimal medium on a gyratory shaker at 250 rpm. Selected mutants were stored as 15% (v/v) glycerol cultures at −20 °C. For further physiological characterization, frozen glycerol stock cultures were directly used for inoculation. Based on the observation that transposon insertion does in most cases not affect riboflavin titers, the average riboflavin concentration of all 96 cultures was used as the plate-specific reference value for identification of relevant mutants.

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 g/L 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 YE (Difco), 27.3 g/L glucose $\cdot H_2O$, 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 $\cdot H_2O$ 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. Identification of Tn10 insertion site

Genomic DNA of interesting mutants was isolated with a commercial kit (Promega, USA). Twenty microliters of DNA isolation mix was digested with *Eco*RI or *Hin*dIII and religated overnight at 16 °C. The ligation mix was used for heat shock transformation of *Escherichia coli* DH5α. Plasmids were isolated from clones and used for sequencing with primer Tn+1 (5′-ttg cat gct tca aag cct g-3′). The obtained sequences were aligned with the *B. subtilis* genome database (http://genolist.pasteur.fr/SubtiList/) to identify the location of the transposon in the genome.

2.4. Analytical techniques

Cell growth was monitored by measuring the optical density at 600 nm (OD_{600}). Cellular dry weight (CDW) was inferred from OD_{600} measurements with a predetermined correlation factor of 0.37 g CDW/L per OD_{600} unit. Glucose and acetate concentrations were determined enzymatically with commercial kits (Beckman, Enzytec, and Boehringer Mannheim). For riboflavin measurements, culture supernatant was diluted with 0.2 M NaOH to the linear range of the spectrophotometer and the absorbance at 440 nm was recorded.

For enzymatic determination of raffinose concentrations (Hessels et al., 2003), cell-free supernatants of liquid cultures were diluted 3:1 with 1 U/mL α-galactosidase (Sigma-Aldrich) solution. Upon 3 h incubation at 37 °C, the concentration of sucrose was determined with a commercial kit (Dispolab). The raffinose concentration was then calculated from the difference in sucrose concentration between α-galactosidase treated and untreated aliquots. Yields and specific consumption and production rates were calculated as described previously (Sauer et al., 1999).