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Selection of quiescent *Escherichia coli* with high metabolic activity

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Abstract

Sustained metabolic activity in non-growing, quiescent cells can increase the operational life-span of bio-processes and improve process economics by decoupling production from cell growth. Because of the ill-defined molecular nature of this phenotype, we developed selection protocols for the evolution of quiescent *Escherichia coli* mutants that exhibit high metabolic activity in ammonium starvation-induced stationary phase. The best enrichment procedures were continuously or discontinuously fed ammonium-limited chemostat cultures with a very low dilution rate of 0.03 h^{-1}. After 40 generations of selection, improved mutants with up to doubled catabolic rates in stationary phase were isolated. The metabolically most active clones were identified by screening for high specific glucose uptake rates during ammonium starvation-induced stationary phase in deep-well microtiter plates.

Keywords: Chemostat selection; Evolution; Metabolic activity; Metabolic engineering; Stationary phase

1. Introduction

A fundamental problem of microbial production processes is channeling of energy and nutritional resources away from the desired product toward biomass formation. Consequently, product yields are typically below the theoretical maximum, which, in turn, affects economic viability of low-value biochemical or bulk protein production with high raw material costs (Chotani et al., 2000). Sustained production in the absence of growth would therefore be potentially valuable to improve productivity and operational live-span of microbial biocatalysts, and genetic (Matin, 1994) or immobilization (Swope and Flickinger, 1996) approaches were proposed. Unlike mammalian cells such as hepatocytes that can acquire a natural state of quiescence (non-growing but metabolically active) (Ilyin et al., 2003), microorganisms enter a metabolically (more or less) inert state upon entry into stationary phase to ensure survival under adverse environmental conditions (Chesbro et al., 1990; Hengge-Aronis, 1996; Nyström, 1998; Venturi, 2003).

Due to the complexity of stationary phase regulation, genetic reprogramming of microbial cells into a state of quiescence with high metabolic activity is not straightforward, but some metabolic engineering success was reported. In growth-arrested Chinese hamster ovary cells, sustained protein production for up to 7 days could be achieved by genetic (Mazur et al., 1999) or environmental modifications (Kaufmann et al., 1999). A similar metabolic engineering strategy for cell cycle arrest was described in *Escherichia coli*: overexpression of Rcd RNA (regulator of cell division) in a *hns* mutant (Rowe and Summers, 1999). Despite the apparent success of accumulating a model protein to more than 40% of total cell protein, continuously declining production could be sustained for only 10 h. In an alternative approach, we had earlier isolated an *E. coli* mutant from a long-term, glycerol-limited chemostat culture (Weikert et al., 1997) that exhibited a partial uncoupling of metabolic activity from growth (Weikert et al., 1998).

As a consequence of the ill-defined molecular nature of complex cellular properties, including quiescence,

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rational metabolic engineering focuses typically on individual biosynthesis or degradative pathways (Chotani et al., 2000; Koffas et al., 1999), particular physiological features (Nielsen, 2001), or on cofactor metabolism in a general sense (Sauer et al., 2004; Vadali et al., 2004; Zamboni et al., 2003). Evolutionary approaches, on the other hand, were particularly successful to improve complex phenotypes (Rohlin et al., 2001; Sauer, 2001; Stephanopoulos, 2002). Recent examples include novel catabolic activities (Arensdorf et al., 2002), incorporation of otherwise toxic amino acid analogues (Bacher and Ellington, 2001), multiple metabolic adjustments (Becker and Boles, 2003; Kuyper et al., 2004; Sonderegger and Sauer, 2003; Sonderegger et al., 2004; van Maris et al., 2004), enhanced production capabilities (Stafford et al., 2002), and improved resistance to environmental stresses, either by long-term selection (Selifonova et al., 2001; Steiner and Sauer, 2003) or genome shuffling (Patnaik et al., 2002). Additional momentum for such evolutionary approaches comes from powerful genomics methods that enable identification of the molecular basis of relevant phenotypes for inverse metabolic engineering (Bro and Nielsen, 2004; Gill, 2003; Gill and Dodge, 2004; Sauer, 2001).

The fundamental problem addressed here was development of a selection strategy based on proliferating cells that enriches for variants with high metabolic activity in the absence of growth, with the inherent goal to develop a true catalyst that does not waste resources for replicating itself. Therefore, we test several selection protocols and developed a robust screening protocol that allows to identify the best clones. Because nutrient starvation-induced stationary phase is an attractive, cost-effective process condition that could be implemented in industrial-scale processes, we focused on glycerol- and ammonium-limited conditions.

2. Material and methods

2.1. Media

The standard minimal medium (pH 7.0) contained per litre: 19 mM NH_4Cl, 8.5 mM NaCl, 53 mM $Na_2H_2PO_4$, 22 mM KH_2PO_4, 0.1 mM $CaCl_2$, 2 mM $MgSO_4$, 1.3 μM thiamine, 0.6 mM $FeCl_3$, 6.3 μM $ZnSO_4$, 7 μM $CuCl_2$, 7.1 μM $MnSO_4$, 7.6 μM $CoCl_2$, 0.6 mM EDTA, and the indicated carbon source. Aerobic, 50 ml batch cultures were grown in 500 ml baffled shake flasks at 37 °C and 200 rpm. Solid media were prepared by adding 1.5% (w/v) technical agar to the medium. Selection chemostat cultures of 1 L were grown in 2 L stirred tank reactors (Bioengineering, Wald, Switzerland) at 37 °C and a dilution rate (D) of 0.03 h^{-1} with glycerol as the sole carbon source. The pH was maintained at 7.0 by automatic addition of 2 N NaOH, and aeration was achieved by an air flow of 1 L min^{-1} and stirring at 1000 rpm. For carbon-limited cultures, the above minimal medium containing was supplemented with 5 g L^{-1} glycerol and the phosphate content was reduced to 34 mM NaH_2PO4. For ammonium-limited cultures, the same reduced phosphate content was used but 15 g L^{-1} glycerol and only 6.5 mM NH_4Cl. Where indicated, discontinuous feeding at an overall D of 0.03 h^{-1} was achieved by adding 5 ml medium every 6 min with an automatic dispenser (Biospectra, Schlieren, Switzerland). Frozen stocks were prepared from overnight LB cultures by adding glycerol to a final concentration of 15% (v/v) and were stored at −80 °C. To preserve original clonal compositions, aliquots of selection cultures were directly supplemented with glycerol and frozen.

2.2. EMS mutagenesis

To increase genetic variability, MG1655 was randomly mutagenized with ethyl methane sulfonate (EMS). For this purpose, a 5 ml LB culture aliquot with an optical density at 600 nm (OD_{600}) of 0.5 was washed twice with LB medium (5000 rpm and 4 °C for 5 min) and resuspended in 2.5 ml LB. Upon addition of 35 μl EMS, the culture was incubated for 2 h at 30 °C and washed twice with 5 ml LB. The pellet was resuspended in 2 ml LB medium, supplemented with glycerol, and stored at −80 °C for further use. About 10% of the cells survived his procedure.

2.3. Screening method

To identify clones with increased metabolic activity, colonies picked from plate were grown in deep-well microtiter plates in a rotary shaker at 30 °C and 300 rpm (Kühner AG, Birsfelden, Switzerland) (Duetz et al., 2000). Four separate 1 ml cultures of each clone were grown in ammonium-limited minimal medium that contained 9 mM NH_4Cl (instead of the above 19 mM) and was supplemented with 100 mM 2-(*N*-morpholino)-ethanesulfonic acid buffer (pH 7.0) and 15 g L^{-1} glucose. OD_{600} and glucose concentrations were determined in traditional flat-bottom 96-well plates with a SpectraMaxPlus plate photometer (Molecular Devices, Sunnyvale, CA) and, for glucose, a commercially available enzymatic kit (Boehringer Mannheim).

Cellular dry weight (DW) was determined as described before (Sonderegger and Sauer, 2003) to determine OD_{600}-to-DW correlations. The specific glucose uptake rate during stationary phase was determined as the ratio of the linear regression coefficient of glucose concentration versus time and the average biomass concentration.