Journal of Biotechnology 158 (2012) 259–266

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Predicting the adaptive evolution of *Thermoanaerobacterium saccharolyticum*☆

Pornkamol Unrean, Friedrich Srienc*

Department of Chemical Engineering and Materials Science, and BioTechnology Institute, University of Minnesota, 240 Gortner Laboratory, 1479 Gortner Ave., St. Paul, MN 55108, USA

ARTICLE INFO

Article history:
Received 1 September 2011
Received in revised form
17 December 2011
Accepted 22 December 2011
Available online 10 January 2012

Keywords:
Metabolic pathway analysis
Adaptive evolution
Elementary modes

ABSTRACT

A fully evolved metabolic network can be described as a weighted sum of elementary modes where the usage probabilities of modes are distributed according to the Boltzmann distribution law (Srienc and Unrean, 2010). An organism presumably achieves the fully evolved state through adaptive changes in the kinetics of rate-controlling enzymes. Metabolic control analysis identifies reactions catalyzed by such enzymes. Comparison of the experimentally determined metabolic flux distributions of *Thermoanaerobacterium saccharolyticum* AS411 with the predicted flux distribution of a fully evolved metabolic network identified phosphoglucose isomerase (PGI) as the enzyme with the greatest flux control, the rate-controlling enzyme. The analysis predicts that an increased activity of PGI would enable the metabolic network to approach the fully evolved state and result in a faster specific growth rate. The prediction was confirmed by experimental results that showed an increased specific activity of PGI in a culture of strain AS411 that adaptively evolved over 280 generations. Sequencing of the gene confirmed the occurrence of a group of mutations clustered in the subunit binding domain of the dimeric enzyme. The results indicate that the evolutionary path is predictable as the strain AS411 adapted toward the fully evolved state by increasing the PGI activity. This experimental finding confirms that enzymes with predicted highest metabolic flux control are the targets of adaptive metabolic pathway evolution.

1. Introduction

Elementary modes are unique, indivisible, balanced pathways that are possible under steady-state operation of the metabolism. They are considered to be fundamental reaction pathways embedded in a metabolic network (Schuster et al., 1999, 2000; Stelling et al., 2002). The overall metabolic network of an organism is represented by an ensemble of elementary modes where individual modes contribute to the network with certain probabilities. An organism evolves by redistributing elementary mode probabilities to reach a fully evolved network. Such network operates based on a distribution of elementary modes using preferentially elementary modes with small reaction entropies in analogy to the preference for low energy states in a physical system that can exist at discrete energy levels (Srienc and Unrean, 2010; Unrean and Srienc, 2011; Wlaschin et al., 2006). The fully evolved network represents the network structure that provides optimal cell growth under selective conditions imposed by the culture conditions (Fong and Palsson, 2004; Hua et al., 2006; Ibarra et al., 2002; Schuster et al., 2008). Using the ensemble method of statistical thermodynamics as applied to the steady state condition of an open system, we have theoretically established that the most probable metabolic state is based on a network with usage probabilities of elementary modes distributed according to an exponential distribution law resulting in the maximum rate of entropy generation (Srienc and Unrean, 2010; Zhao and Kurata, 2009, 2010). Such distribution has been previously suggested by experimental observation and seen in many flux data reported in experimental studies (Zhao and Kurata, 2010). We have recently demonstrated in adaptive evolution experiments that the metabolic network structure indeed evolves toward such predicted state (Unrean and Srienc, 2011). We provide here further evidence for the validity of the presented theory as we show that the mutations occurring during adaptive evolution can be identified based on the predicted pathway structure and the state of the un-evolved cells.

Adaptive evolution is based on mutation and natural selection, and it is expected that an organism achieves a fully evolved network primarily through genetic modifications that result in adaptive alterations in the kinetic parameters of rate-controlling enzymes within the metabolic network (Cork and Purugganan, 2004; Flowers et al., 2007; Vitkup et al., 2006). This could be achieved by direct mutations of specific enzymes enhancing their

☆ Presented in part at the ESF-BU-CeBiTec Conference "Microbes and Industrial Biotechnology 2010", Bielefeld, DE, November 21–24, 2010.

* Corresponding author. Tel.: +1 612 624 9776; fax: +1 612 625 1700.
E-mail address: srienc@umn.edu (F. Srienc).

0168-1656/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jbiotec.2011.12.019

specific activity or through indirect effects that enhance expression levels of enzymes resulting in increased rates. The identification of these rate-controlling enzymes is a challenging problem that requires knowledge of steady-state flux distributions before and after the genetic changes take effect. Because the final state can be predicted and the current state can be measured (Srienc and Unrean, 2010; Unrean and Srienc, 2011) it is possible to apply metabolic control analysis (MCA) and compare the current state with the predicted final state to identify the most critical rate controlling reaction steps (Fell, 1997; Heinrich and Schuster, 1996, 1998; Kacser and Burns, 1979). Rate-controlling enzymes in the networks are identified as the enzyme with maximal flux control coefficients. Both EMA and MCA have been proven to serve as useful guidelines for the optimization and manipulation of cell metabolic pathways (Moreno-Sanchez et al., 2008; Trinh et al., 2009). Recently, a method has been proposed to identify constraining rate controlling reactions based on elementary modes and the assertion that the dissipation function is maximized (Bordel and Nielsen, 2010). However, the dissipation function contains both enthalpic and entropic effects of reactions. Therefore, it is unclear how to consolidate the proposed approach with a consistent physical interpretation since it has been shown that an open isothermal system at steady state depends only on the entropic contributions of the reactions (Unrean and Srienc, 2011). The magnitude of computed metabolic flux control coefficients indicate the steps in the metabolic network that have the strongest effect on cell growth rates. Thus, it is expected that these steps are the most likely targets for mutation during adaptive evolution that selects for fastest growth.

In this study, we applied the Boltzmann distribution law of elementary modes to determine the flux distribution of the fully evolved network of *Thermoanaerobacterium saccharolyticum* AS411 (Unrean and Srienc, 2011). This flux distribution is predicted to represent the most probable metabolic state of strain AS411 with maximal cell growth on glucose under anaerobic conditions. We determined flux control coefficients by comparing the flux distributions of the AS411 network with the predicted fully evolved network. The control coefficients quantify the extent of change in cell growth rate in response to the change in reaction rates of the AS411 metabolism approaching the predicted rates of the fully evolved network. Based on the control coefficients, we identified phosphoglucose isomerase (PGI) as the rate-controlling enzyme with the most significant control on rate of cell growth. Thus, the PGI is predicted as the target of optimization during adaptive metabolic pathway evolution of AS411. The AS411 metabolic network is expected to adapt toward the fully evolved network by increasing the reaction rate catalyzed by this enzyme. The model predictions were confirmed by enzymatic assays that showed an increase in specific activity of PGI in the evolved culture of AS411 that had undergone adaptive evolution under anaerobic growth on glucose for 280 generations. The observed higher PGI activity suggests that evolution and natural selection target the enzymes with the greatest flux control. Furthermore, the results provide further support for the validity of the predicted flux distribution.

2. Materials and methods

2.1. Metabolic evolution

T. saccharolyticum knockout mutant AS411 (Δ*zwf* Δ*mgs* Δ*ldh* Δ*pta* Δ*ack*) which operates using four family modes under anaerobic growth on glucose (Unrean and Srienc, 2011) was evolved in a serial dilution experiment. The evolution experiment was conducted in anaerobic shake tubes (Bellco Glass, Inc., Vineland, NJ) containing MTC medium (10 g/l yeast extract, 5 g/l tryptone, 2 g/l $C_6H_5O_7K_3{\cdot}H_2O$, 1.25 g/l $C_6H_8O_7{\cdot}H_2O$, 1 g/l Na_2SO_4, 1 g/l KH_2PO_4, 2.5 g/l $NaHCO_3$, 5 g/l CH_4N_2O, 1 g/l $MgCl_2{\cdot}6H_2O$, 0.2 g/l $CaCl_2{\cdot}2H_2O$, 0.1 g/l $FeCl_2{\cdot}4H_2O$, 1 g/l cysteine–HCl, 1 mg/l resazurin) and 20 g/l of glucose at 55 °C with no pH control. At each serial dilution step, the cell culture was allowed to grow to exponential growth phase with an approximate optical density of 0.8–1.0 before transferring. The optical density was measured at a wavelength of 600 nm using a Hewlett Packard 8453 Diode Array spectrophotometer (Palo Alto, CA). After 280 generations of serial dilutions, the culture designated AS411E3 was preserved as frozen stock. Individual clones were isolated by plating the evolved cultures on the same medium supplemented with 12 g/l bacto agar.

2.2. Cell growth experiment

Growth experiments were performed either in an anaerobic pH-controlled bioreactor (Biostat MD, B. Braun Biotech International, Melsungen, Germany) with 1 l working volume or in an anaerobic shake tube with 20 ml working volume containing MTC medium and 20 g/l glucose. Cell inoculum was an overnight culture grown in the same medium. The bioreactor experiment was carried out at 55 °C with agitation speed set at 100 rpm. The pH was controlled at 6.0 using 6 M NaOH (Sigma, St. Louis, MO). Specific growth rate was determined from the slopes of linear regression of semi-log time plots of optical density $OD_{600\,nm}$ during the exponential growth phase. Culture samples were also collected for analysis of rate of glucose consumption and product secretion.

2.3. Metabolic flux distribution

Steady-state flux distributions in a metabolic network can be expressed as a linear, weighted combination of the rate of individual reactions specified in each elementary mode. The rates in each elementary mode vector are usually expressed relative to the glucose uptake rate which is set to one. The overall fluxes R_j for the j individual reactions within the metabolic network can be written as

$$R_j = \sum_i p_i r_{i,j} \tag{1}$$

where $r_{i,j}$ represents the rate of reaction j of elementary mode i; p_i is the probability of the elementary mode i contributing to the overall metabolic flux R_j. The sum of all the probabilities equals to one. It is useful to focus on the rates of external metabolite production/consumption since only external metabolites appear in the overall stoichiometry of an elementary mode. Furthermore, these rates can be easily measured. Elementary modes that have the same overall stoichiometry can be grouped into a family of modes (Wlaschin et al., 2006). Thus, Eq. (1) can be written in a reduced form for the net accumulation rates of external metabolites:

$$R_j^{ext} = \sum_k p_k r_{k,j}^{ext} \tag{2}$$

where R_j^{ext} is the net accumulation rate of external metabolites, p_k is the probability for the family mode k, and $r_{k,j}^{ext}$ is the rate of external metabolite accumulation per rate of glucose uptake in the family mode k. Note, this rate is the same for all elementary modes that are part of a family of modes. Eq. (2) allows for the determination of family probabilities from the accumulation rates of external metabolites measured in the experiment. The number of unknown probabilities is significantly reduced through the existence of such mode families. In our case the system of equations was completely determined and the exact solution for the unknown probabilities was possible (Unrean and Srienc, 2011). If an equal, average