Communication to the Editor

Application of the Cross-Regulation System as a Metabolic Switch

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The ability to switch metabolic flow from one pathway to another at a desired point in a bioprocess expands the horizons of metabolic engineering. Such an externally inducible switch can be realized by embedding synthetic operons behind two cross-regulated promoters. This results in coordinated cessation of transcription of one operon while transcription of a second operon is simultaneously activated. The ability to effect such coordinated and inverse control of transcription of two operons has been illustrated experimentally using a model construct containing two different reporter genes, *Vitreoscilla* hemoglobin (VHb) and chloramphenicol acetyltransferase (CAT), fused to λP_L and *tac* promoters, respectively, along with corresponding repressor genes in a cross-regulation configuration. Only VHb production was observed preinduction, and postinduction only CAT was produced. The framework presented here and its obvious extensions can be used with different combinations of promoter systems and synthetic operon constructs to achieve complicated metabolic flux regulation in diverse hosts. © 1994 John Wiley & Sons, Inc.
Key words: metabolic switch • cross-regulation • metabolic flux regulation

INTRODUCTION

The metabolic activities of living organisms are composed of highly regulated, coupled networks of reactions catalyzed by specific enzymes. These naturally occurring networks are not generally optimized for practical applications. However, the performance of these organisms can be artificially improved by genetic manipulations of their metabolic networks. The ability to control flux distribution is a generally important objective. Current approaches usually involve transforming the host with genes that encode for synthesis of the desired products. This strategy, however, does not consider that such extreme flux alterations could be detrimental to host metabolism which is also important for success of the bioprocess. For this reason, the ability to adjust flux distribution from one configuration to another under external control is important. This could be achieved by controlling the relative synthesis of the enzymes catalyzing different pathways.

In this article, a new expression system concept is presented which can serve as a basis for a metabolic switch. The term *metabolic switch* is defined here as the externally inducible redirection of metabolic flux from one pathway to another (Fig. 1). The basis for this pathway switching is a novel cross-regulation transcriptional regulation system[2,3] which provides high-level transcription of one operon (designated I in Fig. 1) preinduction and sharp repression of that operon's transcription postinduction whereas a second operon (II) is strongly activated. Referring to the schematic diagram in Figure 1, this system is expected to provide flux from intermediate B exclusively to metabolite E preinduction. Postinduction, flux from B will switch from the upper pathway to the lower one, forming metabolite F. The switch is expected to occur in a transient fashion as enzyme II accumulates and enzyme I is diluted and/or degraded. Even in the presence of residual enzyme I, overexpression of enzyme II should enable direction of most of the metabolic flux through the lower branch. This phenomenon is well documented in several earlier metabolic engineering studies.[10,11]

Before undertaking experiments with two sets of metabolic genes, the ability of the proposed strategy to switch from expression of one reporter gene to another should be evaluated. This is the purpose of the experiment described here. A naturally occurring example of such a switch in transcriptional activity can be found in *Salmonella* in which a change from flagellin H1 to flagellin H2 takes place in order to evade the immune response of its host.[13] In order to demonstrate the same kind of expression switching using a synthetic genetic construct, the *Vitreoscilla* hemoglobin (VHb) gene[5,6] has been inserted into the vector pKC7, which utilizes the cross-regulation system to coregulate the production of chloramphenicol acetyltransferase (CAT).[3] Transcription of the *vhb* gene is under control of the λP_L promoter and cotranscribed with the *lacI* gene, whereas transcription of the *cat* and *cI* genes is regulated by the *tac* promoter. Verification of the proposed switching of expression of these two genes upon induction behavior provides the bases for future pathway manipulations.

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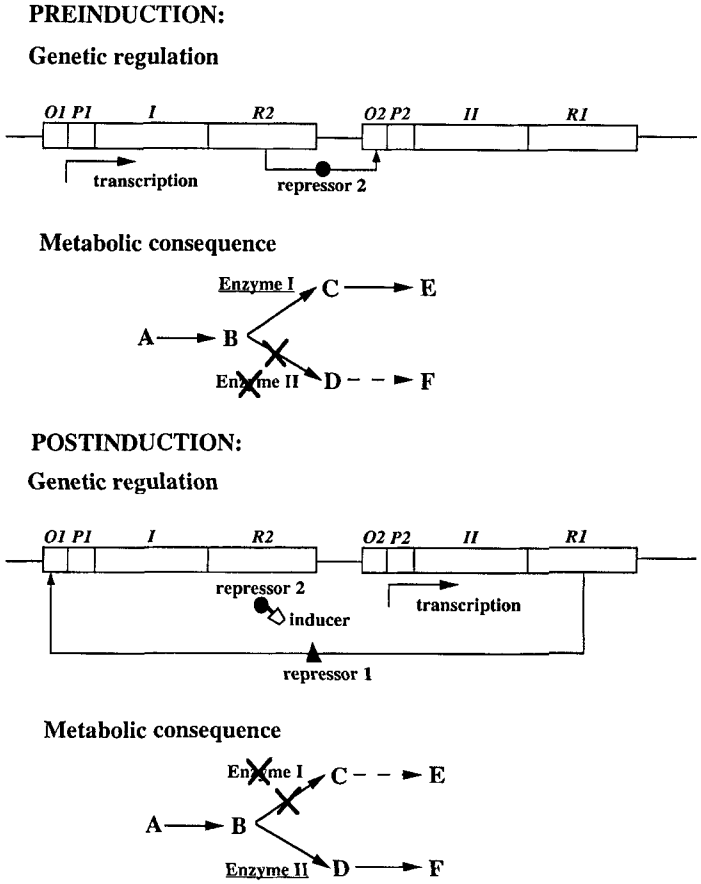

Figure 1. Basic concept of the metabolic switch. Enzymes I and II catalyze the reactions from B to C and from B to D, respectively. In the preinduction state, expression of enzymes I and II is on and off, respectively, providing only the pathway from B to C. After induction, which represses enzyme I expression and induces expression of enzyme II, the pathway from B to D is favored.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli strains DH5α (F^-, *endA1, hsdR17* ($r_k^- m_k^+$), *supE44, thi-1,* λ^-, *recA1, gryA96, relA1,* φ*80dlacAm15*) was used in this work. Plasmid pINT1[7] was used as the source of the *vhb* gene. Plasmids pSL1180 (Pharmacia) and pKC7[3] were used in constructing plasmid pMSW1.

Media and Growth Conditions

For all experiments, Luria–Bertani (LB) medium (10 g/L Difco tryptone, 5 g/L Difco yeast extract, 10 g/L NaCl, 3 g/L K_2HPO_4, and 1 g/L KH_2PO_4, pH 7.0) supplemented with 0.2% glucose was used. Ampicillin, 50 mg/L, was added for selection. Shake-flask experiments were carried out in 250-mL flasks with 100 mL medium at 275 rpm in a New Brunswick INNOVA 4000 incubator shaker at 37°C. For all induction experiments, 1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) was added.

Chemicals, Reagents, and DNA Manipulations

All restriction endonucleases, modifying enzymes (T4 DNA polymerase, Klenow fragment, T4 DNA Ligase), and IPTG were purchases from either New England BioLabs or Boehringer Mannheim Biochemicals. All DNA manipulations were done according to standard methods.[12] The DNA fragments were eluted from agarose gels using a Geneclean Kit (Bio 101).

Immunoblot Analysis of VHb

Cell pellets were boiled for 5 min in a lysis buffer containing 10% glycerol, 5% 2-mercaptoethanol, 3.3% sodium dodecyl sulfate (SDS) and 0.5 *M* Tris, pH 6.8, and then electrophoresced on 12.5% polyacrylamide gel according to the method of Laemmli.[8] The proteins were electrophoretically transferred to a nitrocellulose membrane as described elsewhere.[1] The proteins were screened with antiserum generated against *Vitreoscilla* hemoglobin as described elsewhere.[1] The *Vitreoscilla* hemoglobin standard was produced in recombinant *Escherichia coli.*[7]

Protein and CAT Assays

Cells were disrupted by sonication. The soluble fraction was used for protein and CAT analysis. Protein concentration was determined according to the method of Lowery et al.[9] using a Sigma kit (No. P5656). Total CAT content was measured by a CAT enzyme-linked immunosorbent assay (ELISA) kit obtained from 5 Prime, 3 Prime.

RESULTS

Construction of Plasmid pMSW1

A 1.1-kb *Bam*HI/*Sph*I fragment containing the *vhb* gene was isolated from plasmid pINT1. After gel purification, this fragment was cleaved with *Hae*III, and the resulting 460-bp *Bam*HI/*Hae*III fragment carrying the VHb structural gene without the transcriptional stop signal was subcloned into the *Bam*HI/*Eco*RV sites of pSL1180 to create pSLVHb. This plasmid was subsequently cut with *Bam*HI and filled in with Klenow fragment to generate a blunt end. A 600-bp fragment was isolated by cleaving with *Kpn*I. A *Bsp*EI partial digestion was performed on plasmid pKC7 and the fragment with only one site cut was isolated. This fragment was then cleaved with *Kpn*I and the 600-bp fragment from pSLVHb was inserted to give plasmid pMSW1 (Fig. 2). The resulting construct contains the λP_L-*vhb-lacI* and *tac-cI-cat* operons.

Effect of Induction on VHb and CAT Expression

To investigate the induction response of VHb and CAT production, shake-flask experiments were carried out with strain DH5α/pMSW1. Cultivations were started with a 5% inoculum in order to increase the protein concentration in each sample. Production of CAT and VHb was followed by taking samples at different time points throughout the cultivation. As depicted in Figure 3, a band corresponding to the VHb protein was clearly visible for the sample taken before induction (Fig. 3a, lane 2). This band was not detectable for all subsequent samples which were taken after IPTG addition (Fig. 3a, lanes 3 to 6). On the other hand, production of CAT was not observed from any sample before induction (Fig. 3b). However, after IPTG addition the level of CAT continued to increase and eventually reached 4% of the total soluble protein. In contrast, continuous production of VHb was detected in cultures without IPTG addition (data not shown). These results clearly indicate the desired switching of VHb and CAT production patterns before and after induction.

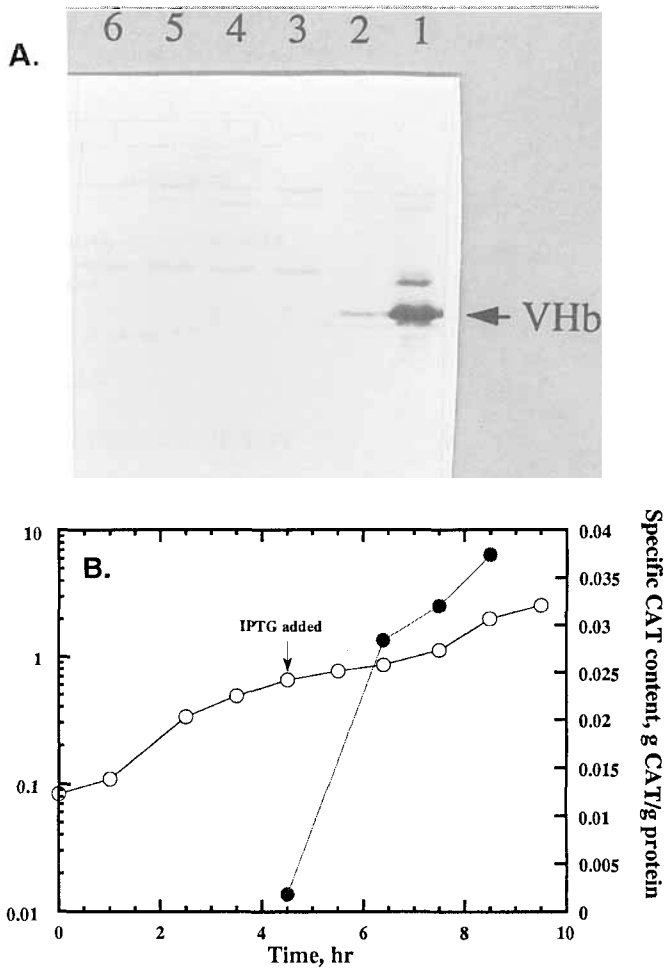

Figure 3. Expression of VHb and CAT from strain DH5α/pMSW1 under noninduced and induced conditions. IPTG was added to the culture after the sampling at 4.5 h postinoculation. (A) Western blot analysis of VHb expression. Samples were harvested at different cultivation times. Lane 1 corresponds to VHb standard. Lanes 2 to 6 correspond to samples at 4.5, 5.5, 6.5, 7.5, and 9.5 h, respectively. (B) Specific CAT content (●) and culture density (○) for DH5α/pMSW1.

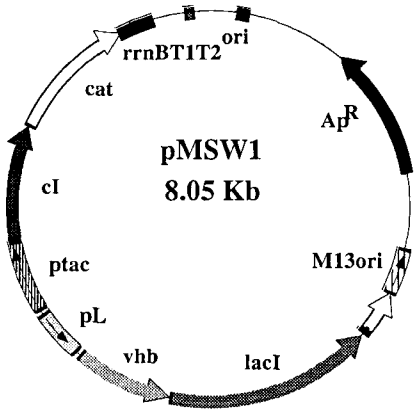

Figure 2. Plasmid map of pMSW1. This is a derivative of pKC7 containing a *vhb* gene inserted between the λP_L promoter and the *lacI* gene. This plasmid contains two separate operons with cotranscription of the *cI* and *cat* genes under control of the *tac* promoter and the *vhb* and *lacI* genes under control of the λP_L promoter.

DISCUSSION

Currently available genetic techniques enable gathering genetic elements from different organisms as well as man-

made or modified genetic components into a single host in order to enhance existing pathways or to construct novel pathways. Achieving desired configurations of active proteins and corresponding metabolic fluxes requires availability of a rich set of regulatory systems for adjusting expression levels both before and during a bioprocess. A contribution to the metabolic engineering tool kit is provided here by formulating a new concept for inducible switching of metabolic activities. The expression switching behavior which is central to this concept has been shown by a model system in which only VHb is produced during the preinduction period and production of VHb and CAT proteins are switched off and on, respectively, postinduction.

This metabolic switch system has recently been applied to coordinate glycogen synthesis and degradation (Dedhia, N., Chen, W., and Bailey, J. E., unpublished results). Using this system, cells are able to synthesize fivefold more glycogen preinduction by overexpressing the glycogen synthesis enzymes. Cells engineered only for glycogen overproduction cannot completely degrade the accumulated glycogen.[4] With the metabolic switch system, however, the glycogen synthesized during the preinduction period has been completely degraded after induction, which switches the cells to overproduction of glycogen-degrading enzyme. It is clear from this example that different metabolic pathways can be switched on and off utilizing the metabolic switch concept. We anticipate that this concept should create new opportunities for more complicated pathway manipulations.

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