Biochemical Engineering Journal 42 (2008) 13–19

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

In vivo monitoring and alleviation of extracytoplasmic stress to recombinant protein overproduction in the periplasm of *Escherichia coli*

Niju Narayanan, Stephanie Follonier[1], C. Perry Chou*

Department of Chemical Engineering, University of Waterloo, 200 University Avenue West, Waterloo, Ontario, Canada N2L 3G1

ARTICLE INFO

Article history:
Received 12 October 2007
Received in revised form 10 March 2008
Accepted 21 May 2008

Keywords:
Chaperone
Inclusion body
Penicillin acylase
Periplasm
Physiology
Stress regulons

ABSTRACT

In *Escherichia coli*, there are two major pathways, i.e. Cpx and σ^E, for dealing with the extracytoplasmic stress in the cell envelope. Due to the unique periplasmic processing steps and the tendency to form periplasmic inclusion bodies, penicillin acylase (PAC) offers a model system for studying the induction of extracytoplasmic stress associated with recombinant proteins overproduction in the periplasm of *E. coli*. In this study, *E. coli* strains carrying the *lacZ* reporter gene fusion with the promoters of three stress-responsive genes, i.e. *degP*, *cpxP*, and *rpoH*, were constructed in the JM109 background for characterization. We demonstrate that *pac* overexpression induced the extracytoplasmic stress primarily via the Cpx pathway. The upregulated *cpxP* promoter activity can be a suitable sensor for in vivo monitoring of the extracytoplasmic stress upon *pac* overexpression. However, such physiological challenge was not observed and all the three promoter activities were reduced when arabinose was used to induce *pac* overexpression. This result suggests that the physiological impact observed for the IPTG (isopropyl-β-D-thiogalactopyranoside)-induced cultures could be overcome by the use of arabinose for induction. The extracytoplasmic stress response associated with *pac* overexpression could be significantly alleviated by the exogenous presence of DegP, but only partially alleviated by its mutant derivative of $DegP_{S210A}$.

1. Introduction

Due to the well-characterized genome, availability of various techniques for genetic manipulation, easy and inexpensive cultivation, *Escherichia coli* remains the most common host for high-level production of recombinant proteins [1]. The cytoplasm is the intracellular compartment where all recombinant proteins are first synthesized and most of them reside. The expressed gene products can be targeted to the extracytoplasmic compartment or even secreted into the extracellular medium. The cell envelope of Gram-negative bacteria surrounds the cytoplasm and is composed of the inner membrane, an aqueous intermembrane space known as the periplasm, the peptidoglycan layer, and the outer membrane [2,3]. In contrast to the cytoplasm, the extracytoplasmic compartment is more viscous and dense with proteins. It can protect cell from the impact of the external environment and maintain cell's integrity. In particular, the periplasm offers several advantages for protein targeting. It consists of only 4% of the total cellular protein and therefore the target protein can be effectively isolated from the majority of cytoplasmic proteins for facilitating downstream protein purification [4]. The oxidizing environment of the periplasm can mediate the formation of disulfide bonds for developing proper protein structure and bioactivity [5,6]. In addition, the in vivo cleavage of the signal peptide upon translocation into the periplasm can yield the authentic N-terminus of the target protein [7]. However, it is common that overexpression of proteins in the extracytoplasmic compartment would generate a local stress in the area of cell envelope, resulting in deterioration in cell physiology and culture performance.

The Gram-negative bacteria have signaling pathways that sense and respond to perturbations within the cell envelope. In *E. coli*, the extracytoplasmic stress is detected via at least two different sensing pathways, the σ^E and the Cpx signal transduction systems [8–10]. The Cpx two-component system consists of the membrane integral sensor histidine kinase CpxA [11] and the cytoplasmic response regulator CpxR [12]. A third periplasmic component CpxP negatively regulates this system [13]. The σ^E envelope stress response is negatively regulated by the antisigma factor RseA and the periplasmic protein RseB [14,15]. Several genes, including *rpoH*, *degP*, *yaeL*, and *fkpA*, etc. [16–18], are regulated by σ^E. Each pathway is activated by a variety of stress signals such as heat shock, overproduction of recombinant proteins, presence of misfolded proteins, high temperature, high pH, and the presence of toxic compounds, etc. Cells respond to these signals by elevated expression of the genes

* Corresponding author. Tel.: +1 519 888 4567x33310.
E-mail address: cpchou@uwaterloo.ca (C.P. Chou).
[1] Present address: ISIC-Institute of Chemical Sciences and Engineering, Ecole Polytechnique Federale de Lausanne, 1015 Lausanne, Switzerland.

1369-703X/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2008.05.017

encoding several proteins that are involved in the degradation and/or renaturation of misfolded periplasmic proteins, including heat shock proteases [10] (e.g. DegP [19]), periplasmic chaperones (e.g. Skp [20,21]), disulfide bond isomerases (e.g. DsbA∼D [22]) and peptydil prolyl isomerases (e.g. FkpA, PpiA [23,20] and SurA [24]. The periplasmic protease/chaperone DegP is induced by both σ^E and Cpx stress regulons [25,16]. From a practical point of view, these proteins could be used for not only quantification but also alleviation of the extracytoplasmic stress.

In our laboratory, various biochemical and genetic strategies have been applied for the production of recombinant penicillin acylase (PAC), an important industrial enzyme for the production of several β-lactam antibiotics [26], in *E. coli*. PAC from *E. coli* serves as an interesting model protein for studying recombinant protein production because the formation of mature PAC in the periplasm involves a series of posttranslational steps, including translocation and periplasmic processing/folding steps [27]. The periplasmic processing mechanism consists of various proteolytic steps via intramolecular autoproteolysis [28,27]. The formation of periplasmic inclusion bodies, which are primarily composed of proPAC, is identified as an important obstacle limiting the overproduction of PAC [29–31]. These insoluble aggregates can seriously deteriorate cell physiology and such impairing effect is host-cell dependent. The presence of exogenous DegP, a periplasmic protein with both protease and chaperone activities, significantly reduces the amount of periplasmic PAC inclusion bodies, suppresses the physiological stress, and, most importantly, increases PAC activity in *E. coli* [32,33]. DegP protease activity is primarily responsible for the improvement via degradation of abnormal proteins in the periplasm when *pac* is overexpressed [33] and this improving effect appears to be also host-cell dependent. All these observations suggest the importance of proper selection of host cell for recombinant protein production.

In this study, we used the *lacZ* reporter gene fusions to the promoters of *degP*, *cpxP* and *rpoH*, respectively, to monitor the extracytoplasmic stress response upon *pac* overexpression which resulted in the formation of insoluble inclusion bodies and severe deterioration of cell physiology [34,35]. Recently, it was demonstrated that arabinose could serve as an effective inducer for the induction of the *lac*-derived promoters for *pac* overexpression in *E. coli* with minimum physiological deterioration [36]. Here, we show that both arabinose induction for *pac* overexpression and the exogenous presence of DegP could relieve the extracytoplasmic stress in PAC-overproducing cells. It appears that the *cpxP* promoter activity could serve as a proper sensor correlating with the physiological stress experienced by the production strain during the course of PAC overproduction.

2. Materials and methods

The *E. coli* strains JM109 (*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*-1 *mcrA* Δ(*lac-proAB*) *F*′[*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]) [37] and their derivatives were used as the hosts for *pac* expression studies. JM594, JM1458 and JM1710 were created by the P1 phage transduction of JM109 with the phage lysate from the derivatives of MC4100 (*F*$^-$ *araD*139 *Δ(argF-lac)U*169 *rpsL*150 *relA1 deoC1 rbsR fthD5301 fruA25* λ$^-$) [38]; i.e. SP594 (MC4100 $\varphi(P_{cpxP}$-*lacZ*)) (Silhavy collection), SR1458 (MC4100 $\varphi(P_{degP}$-*lacZ*) [39] and SR1710 (MC4100 $\varphi(P_{rpoH}$-*lacZ*)) [39]. To prepare the P1 phage lysate from the donor cell, 0.2 mL of an overnight LB (5 g/L NaCl, 5 g/L Bacto yeast extract, 10 g/L Bacto tryptone) culture of the donor strain was infected with the P1 phage and mixed with warm tryptone top agar (10 g/L Bacto tryptone, 5 g/L NaCl, 6.5 g/L Bacto agar, 5 mM $CaCl_2$). This mixture was poured onto a fresh LB agar plate with 5 mM $CaCl_2$ and was incubated at 37 °C until the appearance of plaques. These plaques were incubated with LB broth containing 5 mM $CaCl_2$ overnight at 4 °C. The LB broth containing P1 phage on top of the agar plate was collected, mixed with 0.5 mL chloroform and incubated at 37 °C for 30 min. The supernatant containing the P1 lysate was collected after centrifuging the mixture at 6000 × *g* for 10 min. This P1 phage lysate was later used to infect the recipient cell. To do this, the recipient cells were incubated with P1 lysate in the P1-adsorption medium (5 mM $CaCl_2$, 10 mM $MgCl_2$) at room temperature for 30 min. Then, the mixture was supplemented with LCTG broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1 g/L glucose, 2.5 mM $CaCl_2$) for further incubation at room temperature for 2 h. The cells were later separated from this mixture by centrifugation, resuspended in LCTG broth, and plated on LB agar plate containing 80 μg/mL X-gal for blue–white screening. The efficiency of this P1-transduction protocol appeared to be high enough for transducing alleles into the *recA*$^-$ strains, such as HB101 and JM109, though at a low frequency. pTrcKnPAC2902 [29] contains the *E. coli* wild type *pac* gene encoding preproPAC (S + α + C + β) where S, α, C, and β represent signal peptide, α subunit, connecting peptide and β subunit respectively fused with the *trc* promoter. pARDegP contains the *E. coli degP* gene fused with the *araB* promoter. pARDegP$_{S210A}$ contains the *degP* mutant; *degP*$_{S210A}$ whose expression is under the regulation of the *araB* promoter [33]. The plasmid pTrcKnPAC2902 has a pBR322 replication origin and a kanamycin-resistant (Kn^R) and is therefore compatible with the plasmids, pARDegP and pARDegP$_{S210A}$ derived from pAR3 [40] having a pACYC184 origin and a chloramphenicol (Cm^R) [33]. Plasmid DNA was purified using a spin-column kit purchased from BD Biosciences Clontech. Plasmid transformation was carried out according to Chung and Miller [41].

2.1. Cultivation

The *E. coli* strains were grown in Luria Bertani (LB) broth or 1.5% LB agar at 37 °C. The medium was supplemented with 50 μg/mL kanamycin (Kn) or 34 μg/mL Chloramphenicol (Cm) when necessary. The cultures for protein production were cultivated in Erlenmeyer flasks containing 25-mL LB medium at 28 °C and 200 rpm. When the cell density reached approximately 0.5 OD_{600}, the culture was supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG) and/or arabinose for induction. The flask cultures were further shaken under the same conditions for another 4 h. All the cultivations were conducted in duplicate.

2.2. Cell fractionation

The culture sample at an amount of 20 OD_{600} units (i.e. OD600 × mL) was centrifuged at 6000 × *g* for 6 min at 4 °C to pellet the cells. After discarding the supernatant, the cell pellet was resuspended in 3 mL sodium phosphate buffer (0.05 M, pH 7.5) and lysed by a French Press (ThermoIEC, Waltham, MA, USA). The cell lysate was further centrifuged for 15 min at 12,000 × *g* and 4 °C. The pellet containing insoluble proteins and cell debris was washed with phosphate buffer, resuspended in TE/SDS buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% SDS), and heated to 100 °C for 5 min for dissolution. The supernatant and pellet were analyzed with Western blotting as the soluble and insoluble fractions, respectively.

2.3. Analytical methods

The culture sample was appropriately diluted with saline solution for measuring cell density as the optical density at 600 nm (OD_{600}) with a spectrophotometer (DU®520, Beckman Coulter, Fullerton, CA, USA). The culture supernatant and cell lysate were assayed for the PAC enzyme activity. PAC was assayed at 37 °C