Available online at www.sciencedirect.com
ScienceDirect
Protein Expression and Purification 53 (2007) 264–269
Protein Expression & Purification
www.elsevier.com/locate/yprep

A novel cold-inducible expression system for *Bacillus subtilis*

Ai Thi Thuy Le, Wolfgang Schumann *

Institute of Genetics, Universität Bayreuth, D-95440 Bayreuth, Germany

Received 17 August 2006, and in revised form 19 December 2006
Available online 9 January 2007

Abstract

Production of recombinant proteins at low temperatures is one strategy to prevent formation of protein aggregates and the use of an expensive inducer such as IPTG. We report on the construction of two expression vectors both containing the cold-inducible *des* promoter of *Bacillus subtilis*, where one allows intra- and the other extracellular synthesis of recombinant proteins. Production of recombinant proteins started within the first 30 min after temperature downshock to 25 °C and continued for about 5 h.

Keywords: Cold shock; *Des* promoter; *lacZ*; *htpG*; α-Amylase; Penicillin-binding protein

One of the major drawbacks during high-level production of recombinant proteins in bacteria is the inability of many proteins to reach their native conformation. Under conditions of overproduction, proteins tend to accumulate within refractile aggregates designated inclusion bodies [1]. Since inclusion body formation is believed to arise from the unproductive association of folding intermediates [2], one experimental approach to prevent formation of these aggregates is to increase the intracellular concentration of molecular chaperones to favour "on-pathway" folding reactions and prevent the accumulation of kinetically trapped folding intermediates [3]. An alternative method to limit the aggregation of recombinant proteins consists in cultivating the cells at low temperatures [4]. Besides reducing formation of inclusion bodies, low-temperature expression lowers the degradation of proteolytically sensitive proteins [5,6].

To ensure high level production of recombinant proteins, two different strategies can be used: fusion of the coding region of the protein of interest to an inducible promoter, e.g., using an IPTG- or xylose-inducible promoter [7], or, alternatively, to make use of a cold-shock inducible promoter. When mid-exponential phase bacterial cells are rapidly transferred from 37 to 25 °C or even a lower temperature, the synthesis of most cellular proteins greatly decreases, while that of cold-shock proteins is transiently upregulated [8]. In *Bacillus subtilis*, one of these cold-shock proteins is a membrane-bound desaturase (Δ5-Des) encoded by the *des* gene [9]. This enzyme catalyzes the introduction of a *cis* double bond at the Δ5 position of a wide variety of saturated fatty acids. It has been shown that the *des* gene is tightly regulated during cold shock. While the *des* mRNA is barely detectable at 37 °C, its synthesis is transiently induced upon a temperature downshift [10]. Expression of the *des* gene does not depend on *de novo* protein synthesis, but on a two-component signal transduction system which consists of the sensor kinase DesK and the response regulator DesR [11]. It is assumed that the kinase senses a temperature downshift through changes in the physical state of the cytoplasmic membrane. The C-terminal kinase domain of DesK undergoes autophosphorylation, and the phosphoryl group is then transferred to the response regulator DesR. Phosphorylated DesR binds to two adjacent DNA-binding sites leading to the recruitment of RNA polymerase to the *des* promoter and activation of transcription [12]. The Δ5-desaturase directly introduces double bonds into membrane lipids leading to a return to the original fluidity of the membrane. This is sensed by DesK which changes from a kinase to phosphatase activity

* Corresponding author. Fax: +49 921 552708.
E-mail address: wschumann@uni-bayreuth.de (W. Schumann).

1046-5928/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2006.12.023

leading to a dephosphorylation of DesR with a concomitant turn off of the *des* gene [13].

Based on these data, we developed a cold-inducible expression system for *B. subtilis* making use of the *des* promoter. We show here that cold-induction results in a significant induction of reporter genes largely preventing formation of aggregates of an aggregation-prone protein. Cold-inducible expression systems have also been developed for *Escherichia coli* which are based on a different principle [14,15]. Cold-inducible expression systems provide an inexpensive alternative technology especially for industrial production of recombinant proteins complementing the widely used IPTG- and xylose-inducible systems.

Materials and methods

Materials

Bacteria, plasmids and growth conditions

Bacterial strains and plasmids used are listed in Table 1. Cells were grown in Luria Broth (LB) medium at 37 or 25 °C under aeration. Antibiotics were added where appropriate (ampicillin at 100 μg/ml, neomycin at 10 μg/ml and chloramphenicol at 10 μg/ml).

Methods

Construction of a transcriptional fusion between the des promoter and the lacZ reporter gene

The promoter region of the *des* gene was fused to the *lacZ* reporter gene using the integration vector pDG1728 [16]. The resulting transcriptional fusion is sandwiched between *amyE*-front and *amyE*-back allowing its integration into the *B. subtilis* chromosome at the *amyE* locus. The *des* promoter region was amplified using oligonucleotides (ON) ON1 (GGCCATGAATTCTCCGGCATCCCGATCATCGC; restriction site underlined) and ON2 (GGCCATAAGCTTTCTCATTGTGTGTCTCGGTTCAG). The amplicon was cleaved with *Eco*RI and *Hin*dIII and inserted into pDG1728 cut with the same enzymes resulting in pDG1728-*des*. This recombinant plasmid was transformed into strain WW02, and transformants were selected on LB plates containing chloramphenicol and screened for the loss of the neomycin resistance marker, and one positive transformant (AL03) was kept for further studies.

Construction of a des null mutant

To construct a *des* knockout, the gene including flanking regions was amplified using the primer pairs ON3/ON4 (GGCCATGTCGACTGAACCGAGACACACAATG; GGCCATGAGCTCATAGTTGAGCACCTTTGG), and the amplicon was cleaved with *Sal*I and *Sac*I and cloned into pBluescript SKII$^+$ treated with the same enzymes. Next, the recombinant plasmid was treated with *Hin*dIII and *Bcl*I to remove a 61-bp internal fragment of *des* which was replaced by the *neo* marker using pBgaB as template and the primer pair ON5/ON6 (GGCCATAAGCTTAGGTCGAGATCAGGGAATGAGTT; GGCCATTGATCAGATCAATTCTGACAGCCATG). Using the primer pair ON3/ON4, the modified gene was amplified and transformed into *B. subtilis* 1012. Neomycin-resistant transformants were selected and checked by Southern-blot for chromosomal replacement of the *des* by the *neo* gene (data not shown). One strain (AL02) was kept for further studies.

Table 1
Strains and plasmids used in this study

Strains	Genotype	Reference/source
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr hsdRMS mcrBC*) φ80d *lacZ* Δ*M15 deoR recA1 araD139* Δ(ara leu)$_{7697}$ *galU galK* λ$^-$ *rpsL endA1 nupG*	BRL
B. subtilis		
1012	*leuA8 metB5 trpC2 hsrM1*	[26]
WW02	1012 *amyE*::*neo*	[27]
AL02	1012 *des*::*neo*	This work
AL03	1012 *amyE*::P*des-lacZ*	This work
AL04	AL02 *des*::*neo amyE*::P*des-lacZ*	This work
AL05	AL02 *htpG*::*erm*	This work
AL06	AL02 *pbpE*::*erm*	This work
Plasmids		
pBluescript SK$^+$	Cloning vector	Stratagene
pBgaB	Integration vector containing the *bgaB* gene	[28]
pDG1728	Vector allowing integration of DNA sequences at the *amyE* locus	[16]
pHT01	Derivative of pNDH33 without a direct repeat	[22]
pKTH10	Recombinant vector containing the *amyQ* gene	[17]
pAL10	Expression vector allowing cold-inducible intracellular production of recombinant proteins	This work
pAL12	Expression vector allowing cold-inducible secretion of recombinant proteins	This work
pNDH33-*htpG*	*htpG* fused to an IPTG-inducible promoter	[22]