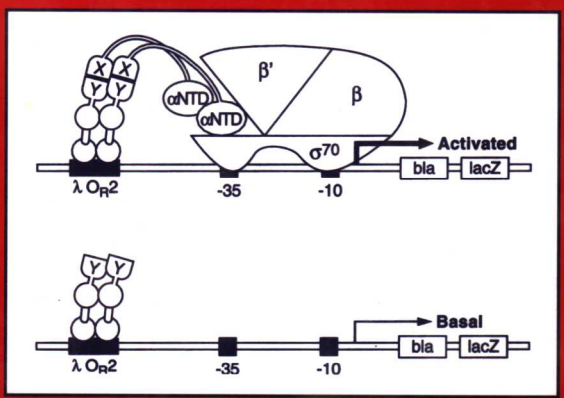
Methods in Molecular Biology™

VOLUME 205

E. coli Gene Expression Protocols

Edited by

Peter E. Vaillancourt



HUMANA PRESS

METHODS IN MOLECULAR BIOLOGY™

E. coli Gene Expression Protocols

Edited by

Peter E. Vaillancourt

Applied Molecular Evolution
San Diego, CA

Humana Press  **Totowa, New Jersey**

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

www.humanapress.com

This publication is printed on acid-free paper. ∞

ANSI Z39.48-1984 (American National Standards Institute) Permanence of Paper for Printed Library Materials.

Cover design by Patricia F. Cleary.

Cover illustration:Principle of a bacterial two-hybrid system (Fig. 1, Chapter 17; *see* full caption and discussion on pp. 251–252).

For additional copies, pricing for bulk purchases, and/or information about other Humana titles, contact Humana at the above address or at any of the following numbers: Tel: 973-256-1699; Fax: 973-256-8341; E-mail: humana@humanapr.com or visit our website at http://humanapress.com

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging-in-Publication Data

E. coli gene expression protocols / edited by Peter E. Vaillancourt.
p. cm. -- (Methods in molecular biology ; v. 205)
Includes bibliographical references and index.
ISBN 1-58829-008-5 (alk. paper)
1. Gene expression--Laboratory manuals. 2. Escherichia coli--Genetics--Laboratory manuals. I. Vaillancourt, Peter E./ II. Series.

QH 450.E15 2003
572.8'65--dc21

2002020572

Preface

The aim of *E. coli Gene Expression Protocols* is to familiarize and instruct the reader with currently popular and newly emerging methodologies that exploit the advantages of using *E. coli* as a host organism for expressing recombinant proteins. The chapters generally fall within two categories: (1) the use of *E. coli* vectors and strains for production of pure, functional protein, and (2) the use of *E. coli* as host for the functional screening of large collections of proteins or peptides. These methods and protocols should be of use to researchers over a wide range of disciplines. Chapters that fall within the latter category describe protocols that will be particularly relevant for functional genomics studies.

The chapters of *E. coli Gene Expression Protocols* are written by experts who have hands-on experience with the particular method. Each article is written in sufficient detail so that researchers familiar with basic molecular techniques and experienced with handling *E. coli* and its bacteriophages should be able to carry out the procedures successfully. As in all volumes of the Methods in Molecular Biology series, each chapter includes an extensive Notes section, in which practical details peculiar to the particular method are described.

E. coli Gene Expression Protocols is not intended to be all inclusive, but is focused on new tools and techniques—or new twists on old techniques—that will likely be widely used in the coming decade. There are several well-established *E. coli* expression systems (e.g., the original T7 RNA polymerase expression strains and vectors developed by William F. Studier and colleagues; the use of GST and polyhistidine fusion tags for protein purification) that have been extensively described in other methods volumes and peer-reviewed journal articles and are thus not included in this volume, with the exception of a few contributions in which certain of these systems have been adapted for novel applications or otherwise improved upon.

It is my sincerest hope that both novice and seasoned molecular biologists will find *E. coli Gene Expression Protocols* a useful lab companion for years to come. I wish to thank all the authors for their excellent contributions and Prof. John M. Walker for sound advice and assistance throughout the editorial process.

Peter E. Vaillancourt

Contributors

ERNESTO ABEL-SANTOS • *Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY*
FRANÇOIS BANEYX • *Department of Chemical Engineering, University of Washington, Seattle, WA*
STEPHEN J. BENKOVIC • *Department of Chemistry, Pennsylvania State University, University Park, PA*
FRANK BREITLING • *Institut für Molekulare Genetik, Universität Heidelberg, Heidelberg, Germany*
PAUL J. BRETT • *Quorex Pharmaceuticals, Carlsbad, CA*
OLAF BRODERS • *Institut für Molekulare Genetik, Universität Heidelberg, Heidelberg, Germany*
MARY N. BURTNICK • *Genomics Institute of the Novartis Research Foundation, San Diego, CA*
DOLORES J. CAHILL • *Max-Planck-Institute for Molecular Genetics, Berlin, Germany; PROT@GEN, Bochum, Germany*
LISA CAMPBELL • *Department of Biochemistry and Biophysics; Center for Macromolecular Design, Texas A&M University, College Station, TX*
CARSTEN-PETER CARSTENS • *Stratagene, La Jolla, CA*
LISA A. COLLINS-RACIE • *Genetics Institute/Wyeth Research, Cambridge, MA*
GREGORY D. DAVIS • *Clontech Laboratories, Palo Alto, CA*
HEIDI A. DAVIS • *The Center for Reproduction of Endangered Species, San Diego, CA*
ELIZABETH A. DIBLASIO-SMITH • *Genetics Institute/Wyeth Research, Cambridge, MA*
SIMON L. DOVE • *Division of Infectious Diseases, Children's Hospital, Harvard Medical School, Boston, MA*
STEFAN DÜBEL • *Institut für Molekulare Genetik, Universität Heidelberg, Heidelberg, Germany*
THOMAS C. EVANS, JR. • *New England Biolabs, Inc., Beverly, MA*

STEVEN M. FIRESTINE • *Department of Medicinal Chemistry, Mylan School of Pharmacy, Duquesne University, Pittsburgh, PA*
JEFFREY D. FOX • *Macromolecular Crystallography Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, MD*
ROGER G. HARRISON • *School of Chemical Engineering and Materials Science, University of Oklahoma, Norman, OK*
SABINE HORN • *Max-Planck-Institute for Molecular Genetics, Berlin, Germany; PROT@GEN, Bochum, Germany*
JAMES C. HU • *Center for Macromolecular Design, Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX*
KARIN A. HUGHES • *Prolinx Inc., Bothell, WA*
JOHN F. HUNT • *Department of Biological Sciences, Columbia University, New York, NY*
ALBERT JELTSCH • *Institut für Biochemie, Justus-Liebig-Universität, Giessen, Germany*
KAREN JOHNSTON • *Department of Biochemistry and Biophysics, The Wistar Institute, Philadelphia, PA*
WOLFGANG KLEIN • *Institute for Pharmaceutical Biology, Rheinische Friedrich-Wilhelm University Bonn, Bonn, Germany*
THOMAS LANIO • *Justus-Liebig-Universität, Institut für Biochemie, Giessen, Germany*
EDWARD R. LAVALLIE • *Genetics Institute/Wyeth Research, Cambridge, MA*
HANS LEHRACH • *Max-Planck-Institute for Molecular Genetics, Berlin, Germany; PROT@GEN, Bochum, Germany*
ZHIJIAN LU • *Genetics Institute/Wyeth Research, Cambridge, MA*
ANGELIKA LUEKING • *Max-Planck-Institute for Molecular Genetics, Berlin, Germany; PROT@GEN, Bochum, Germany*
LEONARDO MARIÑO-RAMÍREZ • *Center for Macromolecular Design, Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX*
RONEN MARMORSTEIN • *Department of Biochemistry and Biophysics, The Wistar Institute, Philadelphia, PA*
JOHN M. MCCOY • *Biogen, Inc., Cambridge, MA*
SAMU MELKKO • *Institute of Pharmaceutical Sciences, Zurich, Switzerland*
MIRNA MUJACIC • *Department of Chemical Engineering, University of Washington, Seattle, WA*
REBECCA L. MULLINAX • *Stratagene, La Jolla, CA*
DARIO NERI • *Institute of Pharmaceutical Sciences, Zurich, Switzerland*

KERSTEIN A. PADGETT • *Division of Insect Biology, University of California, Berkeley, CA*
JOANNE L. PALUMBO • *Department of Chemical Engineering, University of Washington, Seattle, WA*
ALFRED PINGOUD • *Institut für Biochemie, Justus-Liebig-Universität, Giessen, Germany*
FRANK SALINAS • *Department of Chemistry, Pennsylvania State University, University Park, PA*
GERHARD SANDMANN • *Botanisches Institut, Goethe Universität, Frankfurt, Germany*
CHARLES P. SCOTT • *Department of Microbiology and Immunology, Thomas Jefferson University, Philadelphia, PA*
JOSEPH A. SORGE • *Stratagene, La Jolla, CA*
RHESA D. STIDHAM • *Department of Physiology and Graduate Program in Molecular Biophysics, The University of Texas Southwestern Medical Center, Dallas, TX*
PHILIP J. THOMAS • *Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, TX*
DAVID S. WAUGH • *Macromolecular Crystallography Laboratory, Center for Cancer Research, National Cancer Institute, Frederick, MD*
W. CHRISTIAN WIGLEY • *Department of Physiology, The University of Texas Southwestern Medical Center, Dallas, TX*
JEAN P. WILEY • *Prolinx Inc., Bothell, WA*
DAVID T. WONG • *GenVault, Carlsbad, CA*
DONALD E. WOODS • *Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary Health Sciences Centre; Canadian Bacterial Diseases Network, Calgary, Alberta, Canada*
MING-QUN XU • *New England Biolabs, Inc., Beverly, MA*

Contents

1

Cold-Inducible Promoters for Heterologous Protein Expression

François Baneyx and Mirna Mujacic

1. Introduction

1.1. Cold Shock Response and Cold Shock Proteins of Escherichia coli

Rapid transfer of exponentially growing *E. coli* cultures from physiological to low temperatures (10–15°C) has profound consequences on cell physiology: membrane fluidity decreases, which interferes with transport and secretion, the secondary structures of nucleic acids are stabilized, which affect the efficiencies of mRNA transcription/translation and DNA replication, and free ribosomal subunits and 70S particles accumulate at the expense of polysomes, negatively impacting translation of most cellular mRNAs *(1–3)*. It is therefore not surprising that cell growth and the synthesis of the vast majority of cellular proteins abruptly stop upon sudden temperature downshift *(4)*. However, this lag phase is only transient, and growth resumes with reduced rates after 2–4 h incubation at low temperatures, depending on the genetic background *(4,5)*. Such remarkable ability to survive drastic changes in environmental conditions is not atypical for *E. coli,* which has evolved multiple, often synergistic, adaptive strategies to handle stress. In the case of cold shock, the need for restoring transcription and translation is handled by an immediate increase in the synthesis of about 16 cold shock proteins (Csps) *(4)*, while the cell solves the problem of membrane fluidity by raising the concentration of unsaturated fatty acids that are incorporated into membrane phospholipids *(6)*. Interestingly, translation of the alternative sigma factor σ^S, a global regulator of gene expression in *E. coli*, has been reported to increase at 20°C *(7)* suggesting that RpoS-dependent gene products may also play a role in cellular adaptation to mild—but probably not severe—cold shock.

From: *Methods in Molecular Biology, vol. 205, E. coli Gene Expression Protocols*
Edited by: P. E. Vaillancourt © Humana Press Inc., Totowa, NJ

E. coli Csps have been divided into two classes depending on their degree of induction by low temperatures *(8)*. Class II Csps are easily detectable at 37°C but undergo a 2–10 fold increase in synthesis following cold shock. These include the recombination factor RecA, the GyrA subunit of the topoisomerase DNA gyrase, initiation factor IF-2, and HN-S, a nucleoid-associated DNA-binding protein that modulates the expression of many genes at the transcriptional level. By contrast, Class I Hsps are synthesized at low levels at physiological temperatures but experience a more than 10-fold induction following temperature downshift. Two of these, CsdA and RbfA, are associated with the ribosome. CsdA binds to 70S particles and exhibits RNA-unwinding activity *(9)*. RbfA, which only interacts with 30S subunits, has been proposed to function as a late maturation or initiation factor *(2)*, and is required for the efficient translation of most cellular mRNAs at low temperatures *(3)*. Additional Class I Csps include NusA, a transcription termination-antitermination factor, and PNPase, an exonuclease involved in mRNA turnover. The most highly cold-inducible protein, CspA, belongs to a family of nine low molecular mass (≈ 7 kDa) paralogs, four of which—CspA, CspB, CspG and CspI—are upregulated upon temperature downshift with different optimal temperature ranges *(10,11)*. CspA, the best characterized member of the set, has been ascribed an RNA chaperone function based on the observations that it binds single-stranded nucleic acids with low specificity, destabilizes RNA secondary structures *(12)*, and acts as a transcription antiterminator in vivo *(13)*. At present, the function of CspB, CspG and CspI remains unclear, although their high degree of homology to CspA and genetic studies suggests that these proteins may perform similar, albeit complementary roles in cold adaptation *(11,14)*.

1.2. CspA Regulation

CspA, the major *E. coli* cold shock protein, is virtually undetectable at 37°C but more than 10% of the cellular synthetic capacity is devoted to its production during the first hour that follows transfer to 15°C *(15)*. Unlike heat shock genes which rely on specific promoter sequences and alternative sigma factors for transcription, the *cpsA* core promoter is not strikingly different from vegetative promoters (**Fig. 1A**) and is believed to be recognized by the $E\sigma^{70}$ holoenzyme at all temperatures *(16,17)*. An AT-rich UP element, located immediately upstream of the –35 hexamer (**Fig. 1A**) increases the strength of the *cspA* promoter by facilitating transcription initiation *(16,17)*. As a result, large amounts of *cspA* transcripts are synthesized at physiological temperatures. The seemingly inconsistent observation that little CspA is present at 37°C is explained by the presence of a highly structured 159-nt long untranslated region (UTR) at the 5' end of the *cspA* mRNA (**Fig. 1A**; *see* **Note 1**). At 37°C, this extension makes the *cspA* transcript very short-lived ($t_{1/2} \approx 10$ s), thereby preventing its

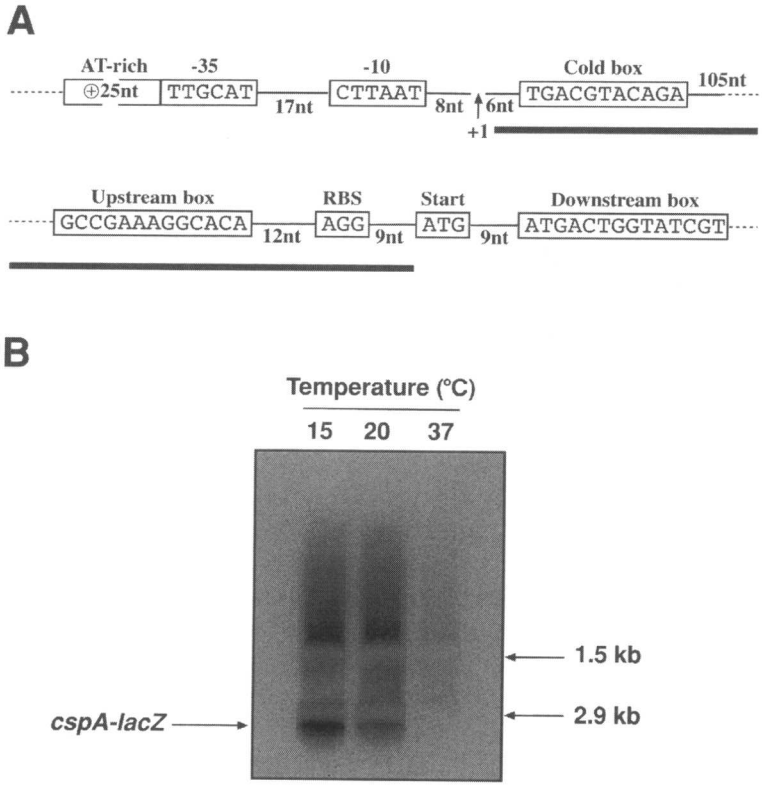


Fig. 1. *cspA* regulatory regions and influence of the downshift temperature on *cspA*-driven transcription. **(A)** Regulatory elements involved in the transcriptional (AT-rich element, -35 and -10 hexamers), posttranscriptional (cold box), and translational (upstream and downstream boxes) control of CspA synthesis are boxed and consensus sequences are given (*see* **Subheading 1.2.** for details). RBS represents the ribosome binding site. The black line spans the length of the 5' UTR. **(B)** JM109 cells harboring pCSBG, a plasmid encoding a *cspA::lacZ* translational fusion ***(21)***, were grown to midexponential phase in LB medium at 37°C and incubated for 45 min at 15, 20, or 37°C. Total cellular RNA was extracted and the *cspA::lacZ* transcript was detected following Northern blotting using a *lacZ*-derived probe. The migration position of the *cspA::lacZ* mRNA and those of the 23S and 16S rRNAs are indicated by arrows (adapted from **ref.** ***(22)***).

efficient translation ***(18–20)***. Of importance for practical applications, the *cspA* UTR is fully portable and fusing it to the 5' terminus of other genes destabilizes the resulting hybrid transcripts at physiological temperatures (*see* **Fig. 1B** and **Note 2** ***[21,22]***).

Following temperature downshift, the *cspA* core promoter is slightly stimulated *(16)* but the main contributor to the rapid induction of CspA synthesis is an almost two order of magnitude increase in transcript stability that appears to be related to a conformational change in the 5' UTR (**Fig. 1B**; *see ref. [18–20,23]*). Translational effects also play a role in the induction process. Deletion analysis indicates that a conserved region near the 3' end of the UTR (the so called upstream box; **Fig. 1A**) makes the *cspA* transcript more accessible to the cold-modified translation machinery *(24)*. In addition, a region complementary to a portion of the 16S rRNA and located 12 bp after the *cspA* start codon (the downstream box; **Fig. 1A**) has been reported to enhance *cspA* translation initiation following cold shock *(17)*. It should however be noted that the latter feature is not essential to achieve efficient low temperature expression of a variety of heterologous genes fused to the *cspA* promoter-UTR region (unpublished data; *see ref. [21,25,26]*).

After 1–2 h incubation at low temperatures, synthesis of native CspA as well as that of recombinant proteins placed under *cspA* transcriptional control stops. An 11 bp-long element located at the 5' end of the UTR and conserved among cold shock genes (the cold box; **Fig. 1A**), as well as CspA itself, appear to be implicated in this process *(27–29)*. It has been hypothesized that the cold box is either a binding site for a repressor molecule or a transcriptional pausing site. In the first scenario, binding of the putative repressor (possibly CspA *[27]*) to the cold box interferes with transcription or destabilizes the mRNA, leading to a shutdown in CspA synthesis. The second model envisions that the putative cold box pausing site is somehow bypassed by RNA polymerase immediately after temperature downshift. However, once CspA reaches a threshold concentration, it binds to its own mRNA, thereby destabilizing the RNA polymerase elongation complex and attenuating transcription *(30)*.

Repression of CspA synthesis coincides with resumption of cell growth. This phenomenon has been explained by the ribosome adaptation model *(3,31)* which states that cold shock proteins RbfA, CsdA, and IF-2 associate with the free ribosomal subunits and 70S particles that accumulate immediately after cold shock to progressively convert them into functional, cold-adapted ribosomes and polysomes capable of translating non cold shock mRNAs. It is possible that these changes in the translational machinery also contribute to the repression of CspA synthesis as suggested by the fact that *rbfA* mutants produce cold shock proteins constitutively following temperature downshift *(3)*. The fact that *rbfA* cells do not repress the synthesis of Csps at the end of the lag phase is of great practical value and has been exploited to significantly increase the intracellular accumulation of gene products placed under *cspA* transcriptional control in both shake flasks and fermentors *(5)*.

1.3. Advantages and Drawbacks of Low Temperature Expression

A number of studies have demonstrated that expression in the 15–23°C range often—but not always—improves the folding of recombinant proteins that form inclusion bodies at 37°C (reviewed in ***ref. [32]***). Although the mechanistic basis for this observation remains unclear, several non-exclusive possibilities can account for improved folding at low temperatures. First, in contrast to other forces (e.g., H-bonding), hydrophobic interactions weaken with decreasing temperatures. Since hydrophobic effects contribute to the formation and stabilization of protein aggregates, newly synthesized proteins may have a greater chance to escape off-pathway aggregation reactions. Second, because peptide elongation rates decrease with the temperature ***(33)***, nascent polypeptides may have a higher probability of forming local elements of secondary structure, thus avoiding unproductive interactions with neighboring partially folded chains. Finally, a decrease in translation rates should increase the likelihood that a protein requiring the assistance of folding helpers to reach a proper conformation is captured and processed by molecular chaperones and foldases based on mass effect considerations.

In addition to improving folding, expression at low temperatures can prove helpful in reducing the degradation of proteolytically sensitive polypeptides (reviewed in ***ref. [32]***). Here again, the fundamental reasons underpinning this phenomenon remain obscure. However, it has been reported that cold shock is accompanied by a transient decrease in the synthesis of heat shock proteins (Hsps; ***[34]***). Since a number of Hsps are ATP-dependent proteases and at least two of these (Lon and ClpYQ) participate in non-specific protein catabolism ***(35)***, a polypeptide synthesized early-on after temperature downshift may have a better chance to bypass the cellular degradation machinery (*see* **Note 3**).

Because aggregation and degradation are two major drawbacks associated with the production of heterologous proteins in *E. coli*, expression at low temperatures is of obvious practical interest. Unfortunately, the vast majority of routinely used promoter systems (e.g., *tac* and T7) experience a decrease in efficiency upon temperature downshift ***(26,32)***. Furthermore, following transfer of cultures to 15°C, the absence of a cold shock UTR precludes translation of typical transcripts by the cold-modified translational machinery until the end of the transient lag phase ***(25)***. Because of its strength and mechanism of induction, the *cspA* promoter-UTR region is particularly well suited for the production of aggregation-prone and proteolytically sensitive polypeptides at low temperatures ***(25,26)***. In addition, by destabilizing elements of secondary structures interfering with ribosome binding, the *cspA* UTR can greatly facilitate the translation of otherwise poorly translated mRNAs. The remainder of this chapter highlights procedures and precautions for *cspA*-driven recombinant protein expression.

2. Materials

2.1. Growth and Maintenance of E. Coli Strains

2.1.1. Strains

1. Routine cloning and plasmid maintenance is in Top10 (F– *mcrA* Δ*(mrr-hsdRMS-mcrBC)* Φ*80* Δ*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ*(ara-leu)7697 galU galK* λ– *rpsL endA1 nupG*) (Invitrogen) or any other *endA recA* strain.
2. A good wild type host for low temperature expression is CSH142 *(5)*.
3. The source of the *rbfA* deletion is CD28 (F' *ara* Δ*(gpt-lac)5 rbfA::kan*) *(2)*.

2.1.2. Growth Media

1. Luria-Bertani (LB) broth: Mix 10 g of Difco tryptone peptone, 5 g Difco yeast extract, and 10 g of NaCl in 950 mL of ddH_2O. Shake to dissolve all solids, adjust the pH to 7.4 with 5 *N* NaOH, and the volume to 1 L with ddH_2O; autoclave. If desired, add 5 mL of 20% (wt/vol) glucose from a filter sterilized stock.
2. LB plates: add 15 g of agar (Sigma) per liter of LB before autoclaving.
3. Add antibiotics at a final concentration of 50 μg/mL after the solution has cooled to 40–50°C (*see* **Note 4**).

2.1.3. Antibiotic Stock Solution

1. Prepare stock solutions of carbenicillin (or ampicillin), and neomycin (or kanamycin) at 50 mg/mL by dissolving 0.5 g of powder into 10 mL of ddH_2O and filter-sterilizing the solutions through a 0.2 μm filter. Antibiotics are stored in 1 mL aliquots at –20°C until needed (*see* **Note 5**).

2.1.4. Glycerol Stock

1. Weigh 80 g of glycerol in a graduated cylinder. Fill to 100 mL with ddH_2O. Sterilize through a 0.2 μm filter into a sterile bottle and store at room temperature.

2.2. Cloning Vectors for cspA-Driven Expression and rbfA Strains

2.2.1. Plasmids pCS22 and pCS24

1. These plasmids are available upon request from François Baneyx, Ph.D., Department of Chemical Engineering, The University of Washington, Box 351750, Seattle, WA 98195.

2.2.2. Construction and Phenotypic Verification of rbfA::kan Mutants

1. CD28 donor strain (*see* **Subheading 2.1.1.**), desired recipient strain and P1*vir* lysate.
2. $CaCl_2$ solution in ddH_2O (1 *M*), filter sterilized and stored at room temperature.
3. Soft agar: Add 0.75 g of agar to 100 mL of LB (*see* **Subheading 2.1.2.**) and autoclave. Dispense 3 mL aliquots in sterile 18 mm culture tubes before solidification. Store tubes at room temperature.

4. LB medium: LB and LB-neomycin plates (*see* **Subheading 2.1.2.**).
5. Chloroform.
6. MC buffer: 100 m*M* $MgSO_4$, 5 m*M* $CaCl_2$ and 1 *M* sodium citrate in ddH_2O. Filter-sterilize both solutions and store at room temperature.

2.2.3. Transformation and Storage of rbfA Mutants

1. $CaCl_2$ in ddH_2O, 100 m*M*, filter sterilized and stored at room temperature.
2. Glycerol stock solution (*see* **Subheading 2.1.4.**).

2.3. Cold Induction in Shake Flasks and Fermentors

2.3.1. Shake Flasks Cultures

1. Temperature controlled water bath with orbital shaking.
2. Cooling coil accessory.
3. VWR model 1172 refrigeration unit or equivalent.

2.3.2. Fermentations

1. New Brunswick BioFloIII fermentor or equivalent equipped with temperature, agitation, pH, dissolved oxygen and foam control.
2. Antifoam (Sigma 289).
3. Glucose stock solution, 20% w/v, filter sterilized.
4. 1 *M* HCl and 5% NH_4OH (v/v) for pH control.
5. Neslab Coolflow HX-200 cooling unit or equivalent.

3. Methods

3.1. Placing PCR Products under cspA Transcriptional Control

The cloning vectors pCS22 and pCS24 (**Fig. 2**; ***ref. [25]***) are pET22b(+) and pET24a(+) (Novagen) derivatives that have been engineered to facilitate the positioning of structural genes downstream of the *cspA* promoter-UTR region. Plamid pCS22 is an ampicillin-resistant construct encoding the ColE1(pMB1) origin of replication, a *pelB* signal sequence, a multiple cloning site (MCS) derived from pET22b(+), a 3' hexahistidine tail, and the phage T7 transcription termination sequence (**Fig. 2A**). Plasmid pCS24 is a kanamycin-resistant ColE1 derivative encoding a MCS derived from pET24a(+), a 3' hexahistidine tail and the phage T7 terminator region (**Fig. 2B**). For cytoplasmic expression, cloning should be carried out as follows:

1. Amplify the desired gene using a forward primer designed to create a *Nde*I site overlaping the ATG initiation codon and a reverse primer selected to introduce one of the unique restriction sites available in the MCS of pCS22 or pCS24 (we typically make use of *Xho*I).
2. Purify the amplified fragment following low melting point (LMP) agarose electrophoresis using the QIAGEN QIAquick gel extraction kit or equivalent. If *Taq*

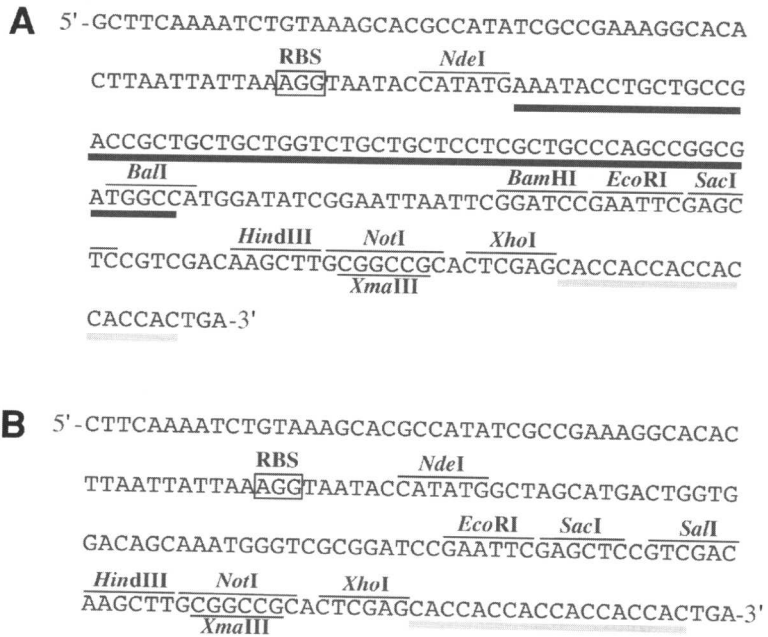


Fig. 2. Cloning regions of pCS22 and pCS24. **(A)** Unique restriction sites in the polylinker of the ampicillin-resistant ColE1 derivative pCS22 are shown. The black line spans the length of the *pelB* signal sequence. The gray line shows the location of the hexahistidine tail. **(B)** Unique restriction sites in the polylinker of the kanamycin-resistant ColE1 derivative pCS24 are shown. The gray line shows the location of the hexahistidine tail. RBS represents the ribosome binding site.

polymerase has been used for amplification, subclone the purified DNA fragment into the Invitrogen TOPO TA cloning vector or equivalent according to the manufacturer's instructions. If the polymerase yields a blunt fragment, subclone into Invitrogen Zero Blunt TOPO cloning vector or equivalent.

3. Digest pCS22 or pCS24 DNA and the plasmid encoding the desired gene with *Nde*I and the appropriate 3' enzyme (*see* **Note 6**). Isolate backbone and insert DNA following LMP agarose electrophoresis as in **step 2**.
4. Ligate at a 3:1 insert to backbone ratio, transform electrocompetent Top10 cells and plate on LB agar supplemented with 50 μg/mL of carbenicillin (for pCS22 derivatives) or 50 μg/mL neomycin (for pCS24 derivatives). Screen the colonies for the presence of the insert.

It is possible to target gene products to the *E. coli* periplasm by taking advantage of the presence of the *pelB* signal sequence in pCS22 (**Fig. 2A**; *see* **Note 7**). However, the *Nco*I site which is typically used to fuse gene products to the *pelB* signal peptide in pET22b(+) is no longer unique in pCS22. Downstream