Preface

Could there be a better time to be a life scientist? In the past two decades, a host of new techniques have been added to the tool chests of biochemists and molecular biologists. A wonderful benefit of the basic scientific research that fueled the advances in these fields is the wide variety of direct applications in agriculture and medicine. Even with all of these advances, and with the accompanying explosion in computer and information technology, it is clear that the depth of our ignorance vastly exceeds the breadth of our knowledge about complex organisms at the molecular level. Any new techniques or materials that allow us to extend our research-based knowledge should be welcomed and utilized to their fullest potentials. With the cloning of the green fluorescent protein (GFP) from *Aequorea victoria* in 1992, another valuable tool was added to the arsenal. In *Green Fluorescent Proteins: Applications and Protocols* examples of how GFP can be utilized in a variety of fields are presented. Although the text has chapters that emphasize different areas of specialization, it is not meant to send molecular biologists to one section, botanists to another, and clinicians to still another. Perhaps the most valuable exchange for people in any discipline will come from seeing how others have been able to apply GFP in fields outside of their immediate areas of expertise.

GFP from *Aequorea victoria* is a fluorescent marker protein, and there are certainly other useful fluorophore markers. The wild-type GFP is not generally used by researchers today. In fact, the acronym GFP has become somewhat misleading because so many spectral variants are now available. All of the work described in this volume takes advantage of the mutant GFPs with altered spectral characteristics or with great cellular expression. It is also noteworthy that the first two chapters describe technique applied to other fluorescent markers: DsRed and other fluorescent proteins cloned from Anthozoans, and *cobA* and *CysG*, genes encoding for enzymes producing soluble red fluorescent markers. Although using the GFP marker to locate biomaterials remains the most often utilized application because of the advantages inherent in using GFP and the versatility offered by the many GFPs available, many more elegant methods have emerged, and several of these are demonstrated in this volume. Like all volumes in the Methods in Molecular Biology series, the text is designed to aid researchers who understand broad aspects of a topic to gain expertise in some narrow experimental portion of that topic. It might be most useful to postdoctoral researchers or graduate students who are actually per-

forming the experimental work at the bench. In each chapter, methods with detail that go far beyond what is currently printed in most journals are provided and could aid in spreading GFP techniques to new laboratories.

Several groups and individuals deserve special attention for getting this text completed. Although the majority of the figures in the text are in black and white, I urge readers to take full advantage of the accompanying CD-ROM that was generously sponsored by Universal Imaging Corporation. The CD-ROM includes color figures and videos from over half of the chapters in this book. I would like to thank Dr. John Walker for allowing me the opportunity to edit this volume and further my own understanding of life science, which also allowed me to make research contacts with some fantastic people around the world utilizing autofluorescent proteins. Finally, I would like to thank my students at the US Air Force Academy for continuing to challenge me to stay abreast of the rapidly advancing discipline of biochemistry.

Barry W. Hicks

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Use of *cobA* and *cysG*^{A} as Red Fluorescent Indicators

Charles A. Roessner

1. Introduction

This chapter is based on the observations ***(1–3)*** that *Escherichia coli* cells bearing the plasmid pISA417, for the overexpression of the *cobA* gene from the bacterium *Propionibacterium freudenreichii*, or the plasmid pEB1, for the overexpression of a truncated *cysG* (*cysGA*) gene of *E. coli*, exhibit bright red fluorescence (**Fig. 1**) when cultured on Luria-Bertani (LB) growth medium and illuminated with ultraviolet (UV) light. The genes both encode uroporphyrinogen III (urogen III) methyltransferases (referred herein to as CobA or CysG^{A}) which catalyze the methylation of urogen III, an intermediate in heme biosynthesis, using *S*-adenosyl-L-methionine as the methyl donor. Plasmid pISA417 was constructed by insertion of a DNA fragment bearing the complete *cobA* gene into pUC19 (**Fig. 2**) and was originally used for the characterization of urogen III methyltransferase ***(1)***. During this study, it was noticed that *E. coli* colonies harboring pISA417 are brightly red fluorescent when illuminated with UV light. However, *E. coli* cells harboring pISA417 bearing a DNA insert that deletes or knocks out the expression of *cobA* are not fluorescent, thus providing the basis for its first use as a fluorescent indicator in selecting recombinant plasmids ***(2)***.

The fluorescence is caused by the cytoplasmic accumulation of two polar fluorescent compounds derived by the methylation of urogen III at C-2, C-7, and C-12 (**Fig. 3**), to afford dihydrosirohydrochlorin (precorrin-2) and a fluorescent trimethylpyrrocorphin ***(1,4)***. Precorrin-2 is oxidized to the fluorescent sirohydrochlorin (factor II) either by oxygen, or enzymatically, by CysG and NAD. In contrast to heme and siroheme, whose cellular concentrations are tightly regulated in *E. coli*, the fluorescent compounds are synthesized and

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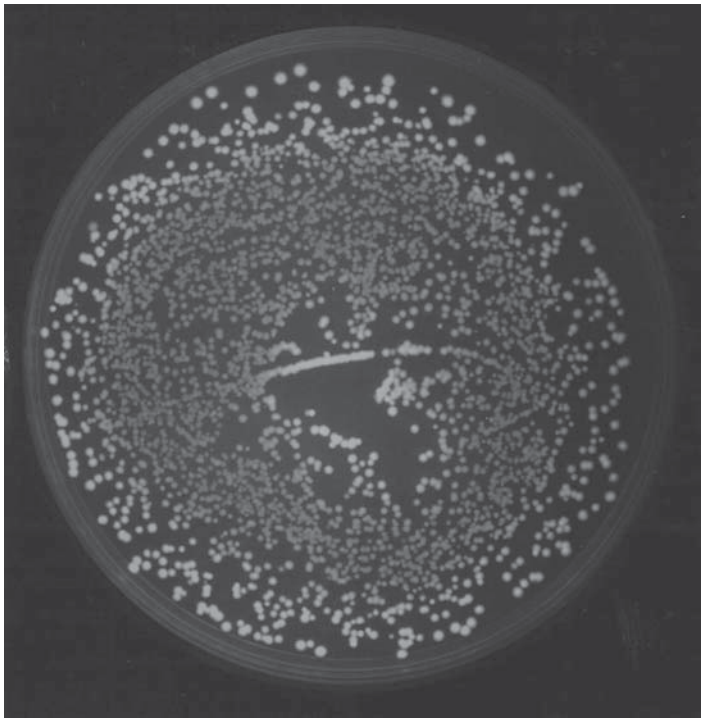


Fig. 1. A red fluorescent strain of *E. coli*. Strain CR417 (TB1 bearing pISA417) was grown on an LB-AMP plate and photographed with UV illumination. (For optimal, color representation please see accompanying CD-ROM.)

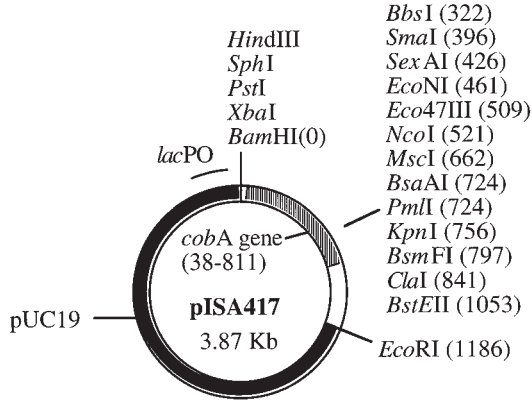
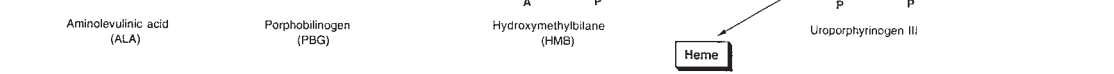
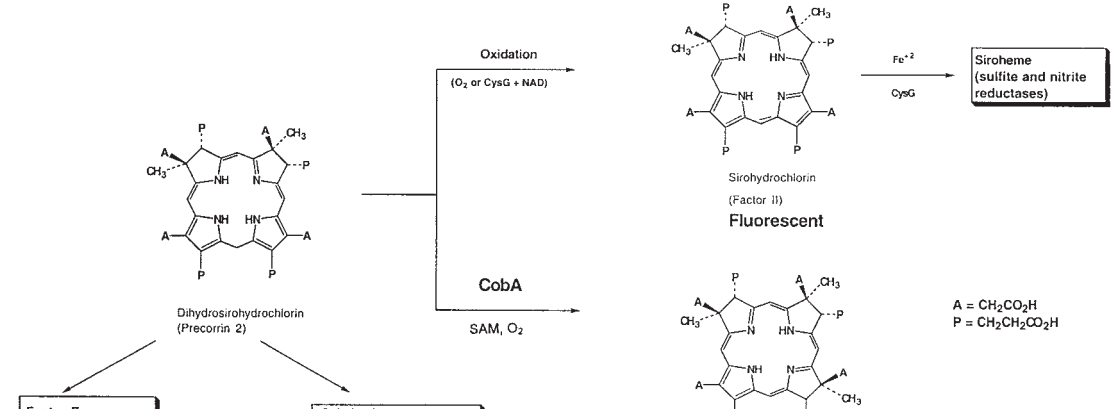


Fig. 2. The structure of pISA417, showing the location of the unique restriction sites derived from pUC19 and a 1.1-kb *Bam*HI-*Eco*RI insert bearing the *P. freudenreichii cobA* gene. The *Cla*I and *Bst*EII sites lie outside the *cobA* gene. The sites within *cobA* were predicted from the sequence (Genbank accession no. U13043). (Also on CD-ROM.)


Aminolevulinic acid
(ALA)
Porphobilinogen
(PBG)
Hydroxymethylbilane
(HMB)
Uroporphyrinogen III
Heme


Oxidation
(O2 or CysG + NAD)
Sirohydrochlorin
(Factor II)
Fluorescent
Fe+2
CysG
Siroheme
(sulfite and nitrite
reductases)
CobA
SAM, O2
Dihydrosirohydrochlorin
(Precorrin 2)
A = CH2CO2H
P = CH2CH2CO2H

accumulate at relatively high levels, probably because of loss of feedback inhibition of aminolevulinic acid (ALA) synthesis in cells overexpressing urogen III methyltransferase ***(5)*** and stability of the products.

In *E. coli* and some other bacteria, such as *Salmonella typhimurium* and *Neisseria meningitidis*, urogen III methyltransferase is part of siroheme synthase (CysG), a multifunctional enzyme encoded by the *cysG* gene. CysG contains not only urogen III methyltransferase activity ($CysG^A$) in its C-terminal region, but also NAD-dependent oxidase and ferrochelatase activities ($CysG^B$) in its N-terminal region, which convert precorrin-2 to siroheme ***(3,6)***. Thus, overexpression of the complete *cysG* gene in *E. coli* leads to accumulation of siroheme, which is not fluorescent.

This chapter describes two methodologies: the use of pISA417, carrying the *cobA* gene as a red fluorescent indicator, for the selection of recombinant plasmids, and a protocol for the expression of the truncated *cysGA* gene. The procedure described for overexpression of *cysGA* in *E. coli* uses the polymerase chain reaction (PCR) and vector selection, to provide strong transcriptional and translational signals. Demonstrating its utility, this procedure has been adapted to construct plasmids for expressing *cobA* from *Pseudomonas denitrificans* and *UMP1* from *Arabidopsis thaliana* ***(5,7)*** to give red fluorescent *E. coli*. Since the *P. freudenreichii cobA* gene is derived from a high G-C, Gram-positive bacterium, it may not be suitable for expression in all organisms, and alternative sources of the gene may be desirable. However, similar technology has recently expanded the use of the *P. freudenreichii cobA* gene as a regulated red fluorescent reporter not only in bacteria but also in yeast (*Schizosaccharomyces pombe*) and cultured mammalian (Chinese hampster ovary) cells ***(8)***. In the latter case, *cobA* was expressed either by itself to provide red fluorescent cells, or in conjunction with the green fluorescent protein, to create cells that emitted both red and green fluorescence.

2. Materials

1. LB medium: 5 g/L yeast extract (Difco), 10 g/L tryptone (Difco), and 5 g/L NaCl.
2. LB agar: LB medium, add 15 g/L agar (Difco) before autoclaving. Add 50 µg/mL ampicillin (Sigma, sodium salt), after autoclaving. Add 10–20 µg/mL aminolevulinic acid (Sigma), after autoclaving, from a 10-mg/mL stock solution of ALA sterilized by filtration (ALA is destroyed by autoclaving).
3. *E. coli* K12 strain TB1 ***(9)*** is used throughout this work (*see* **Note 1**).
4. pISA417 is supplied (*see* **Note 2**) in strain CR417 (TB1 bearing pISA417).
5. pCR252 bearing the *E. coli cysG* gene ***(10)*** is isolated from TB1(pCR252).
6. pUC19 ***(11)*** is isolated from TB1(pUC19).
7. STET buffer: 8% sucrose, 50 m*M* Tris-HCl, pH 8.0, 50 m*M* EDTA, 0.5% Triton X-100; autoclave, and store at room temperature.

8. Egg white lysozyme (Sigma, 10 mg/mL in water).
9. Isopropanol.
10. 70% Ethanol, 100% ethanol.
11. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1.0 m*M* EDTA.
12. The insert DNA can be any DNA fragment of interest, whether a PCR product or a restriction fragment. In the example given here, a 0.6 kb blunt end PCR product is used.
13. Restriction enzymes and buffers: *Bam*HI, *Eco*RI, *Sma*I, and their 10X buffers (New England Biolabs).
14. T4 DNA ligase with 10X buffer (New England Biolabs).
15. *Taq* polymerase, 10X polymerase buffer (Mg free), and 25 m*M* $MgCl_2$ (Promega).
16. dNTPs for PCR (New England Biolabs). Dilute the four dNTPs (100 m*M*) to 10 m*M* with water, then a mixture is prepared by combining 50 µL of each dNTP with 200 µL water (1.25 m*M* final concentration). Store dNTP solutions at –20°C.
17. Phenol pH 8.0 (Ambion) is stored at –20°C. Prior to use, 8-hydroxyquinoline is added to 0.1%.
18. Autoclaved 7.5 *M* ammonium acetate.
19. Chloroform.
20. Sterile 10% glycerol.
21. Electroporation cuvets (1.0-mm gap) and an electroporator, e.g., the *E. coli* Pulser (Bio-Rad).
22. Recovery medium: 50 mL LB broth supplemented with 1.0 mL 20% glucose, 0.5 mL 1.0 *M* $MgSO_4$, and 0.05 mL 1.0 *M* $CaCl_2$.
23. For PCR, the following template and primers were used:
    a. Template DNA. pCR252 containing the complete *cysG* gene (***10***; *see* **Note 3**).
    b. PCR primers were synthesized on the 40-nmol scale. The *Bam*HI, RBS, and start codons are indicated:
    5' primer

    *Bam*HI RBS **Start** codons 211–220

    5'-CGCGCGGATCCAGGAAGGAATTTAAA**ATG***GAA*ACGACCGAAC AGTTAATCAACGAACCG-3'

    3' primer

    *Eco*RI **stop** anticodons 457–448

    5'-CGCCGGAATTC**TTA**ATGGTTGGAGAACCAGTTCAGTTTATCGCG-3'

    A 0.1-nmol/µL stock solution of the primers is prepared by dissolving 40 nmol of the primer in 400 µL of TE buffer, and stored at –20°C. Just prior to use, an aliquot of the stock is diluted to 0.01 nmol/µL with water.

3. Methods

3.1. Selection of Recombinant Plasmids, Using pISA417

The insertion of any DNA fragment into pISA417, as described here, using any of the unique sites shown in **Fig. 2** (*see* **Note 4**) will disrupt the *cobA* gene and result in nonfluorescent colonies.

3.1.1. Isolation of pISA417

1. Strain CR417 is usually received as filter disks that have been saturated with an overnight culture of the strain.
2. To recover the strain, place a filter disk on an LB-amp plate, streak for isolation, and incubate 16–20 h at 37°C.
3. Briefly illuminate the plate with a long-wavelength (302 nm) UV transilluminator (*see* **Note 5**), select a brightly fluorescent colony to inoculate into 50 mL LB-amp, and incubate overnight at 37°C, in a shaking water bath.
4. Fill a 1.5-mL microcentrifuge tube with the culture, pellet the cells in a microcentrifuge, and discard the supernatant. The cell pellet should be brightly fluorescent.
5. Resuspend the cells in 200 µL STET buffer (vortex vigorously), add 20 µL lysozyme solution, mix, and place the tube in a boiling water bath for 40 s.
6. Centrifuge at top speed in a microcentrifuge (≥10,000*g* for all microcentrifugations) for 15 min and remove the viscous pellet with a flat toothpick.
7. Add an equal volume of isopropanol (usually 150–200 µL), mix, and centrifuge for 10 min.
8. Remove the supernatant, add 0.5 mL 70% ethanol, vortex briefly, and centrifuge for 5 min.
9. Remove the supernatant, and dry the pellet under vacuum (Speed-Vac or lyophilizer).
10. Dissolve the pellet in 50 µL TE buffer (vortex vigorously), and store at –20°C. This procedure normally yields DNA concentrations of 100–200 ng/µL.

3.1.2. Restriction Enzyme Digestion

1. For this example, pISA417 is digested with *Sma*I in the following mixture, in a 0.5-mL microcentrifuge tube: 2 µL pISA417 (200 ng), 10 µL insert DNA (10–200 ng), 4 µL 10X *Sma*I buffer,1 µL *SmaI*I (10–20 U), sufficient water (23 µL) to make the total volume 40 µL.
2. Incubate the mixture 1 h at 25°C.
3. Extract the restriction digest with phenol to inactivate the enzymes. Add an equal volume of phenol to the digest and vortex for 1 min.
4. Centrifuge at top speed in a microcentrifuge and transfer the upper layer to a clean 0.5-mL tube. This layer should be clear but will sometimes appear milky, because of precipitation of phenol. The lower (phenol) layer will be yellow from the hydroxyquinoline.
5. To remove dissolved phenol from the DNA solution, add 40 µL chloroform, vortex briefly, centrifuge briefly, and remove the bottom (chloroform) layer with a micropipet. Perform the chloroform extraction a second time.
6. Precipitate the DNA by adding one-half vol 7.5 *M* ammonium acetate and 2 vol 100% ethanol. For example, if there is 30 µL DNA solution remaining after extraction with phenol and chloroform, add 15 µL ammonium acetate and 90 µL ethanol.

7. Mix and place the tube at –80°C for at least 30 min, centrifuge 10 min at top speed in a microcentrifuge, to pellet the DNA, and remove the supernatant.
8. To wash the pellet (usually not visible) add 200 µL 70% ethanol, vortex briefly, centrifuge 5 min, and completely remove the supernatant.
9. Dry the pellet for at least 1 h under vacuum to remove any remaining traces of the volatile ammonium acetate.

3.1.3. Ligation and Ethanol Precipitation of DNA Fragments

1. Dissolve the DNA pellet from **Subheading 3.1.2.** in 17 µL water, add 2 µL 10X ligation buffer, 1 µL T4 DNA ligase, and incubate 16–20 h at 16°C.
2. After ligation, ethanol precipitate the DNA as described in **Subheading 3.1.2.** and dissolve the pellet in 10 µL water.

3.1.4. Transformation of Electrocompetent TB1 Cells by Electroporation and Selection for Recombinant Plasmids

1. Produce electrocompetent cells by inoculating a colony of TB1 into 50 mL LB and incubate overnight at 37°C in a shaking water bath.
2. Inoculate two fresh 50-mL portions of LB with 0.5 mL of the overnight culture and grow the cells to an A_{600} = 0.8 at 37°C. Chill the cultures on ice and pellet the cells at 5000 rpm for 10 min in sterile 50-mL tubes in a Sorvall SS34 rotor or its equivalent. All centrifugations are done at 4°C.
3. Remove the medium and wash the cell pellets twice by gently resuspending them in 20 mL ice cold 10% glycerol and centrifugation as above. After the second wash, resuspend both pellets in a total of 1.0 mL 10% glycerol, and pellet the cells in a 1.5-mL microcentrifuge tube.
4. Resuspend the final pellet in 400 µL 10% glycerol, divide into 50-µL aliquots in microcentrifuge tubes on ice, and store at -80°C.
5. Thaw a tube of the electrocompetent TB1 cells on ice, and mix in 5 µL of the DNA solution.
6. Transfer the mixture to an ice-cold electroporation cuvet (1.0-mm gap), and incubate on ice for 5 min.
7. Thoroughly dry the outside walls of the cuvet, and electroshock the cells, using a setting of 1.8 kV on the electroporator.
8. Immediately add 1.0 mL recovery medium, and incubate the cells for 1.0 h at 37°C, to allow the cells to recover from the shock and allow expression of the ampicillin resistance gene.
9. Plate the cells by spreading on LB-amp plates (*see* **Note 6**) and incubate 16–20 h at 37°C. Several different amounts (1, 10, 100 µL) of cells should be plated, to ensure obtaining a plate that has isolated colonies. The smaller amounts should be added to 100 µL sterile water, before spreading.
10. Examine the plates with long-wavelength (302 nm) UV light (remove the Petri dish cover, and invert the plate over the light source), and select nonfluorescent colonies (**Fig. 4**) for further analysis.

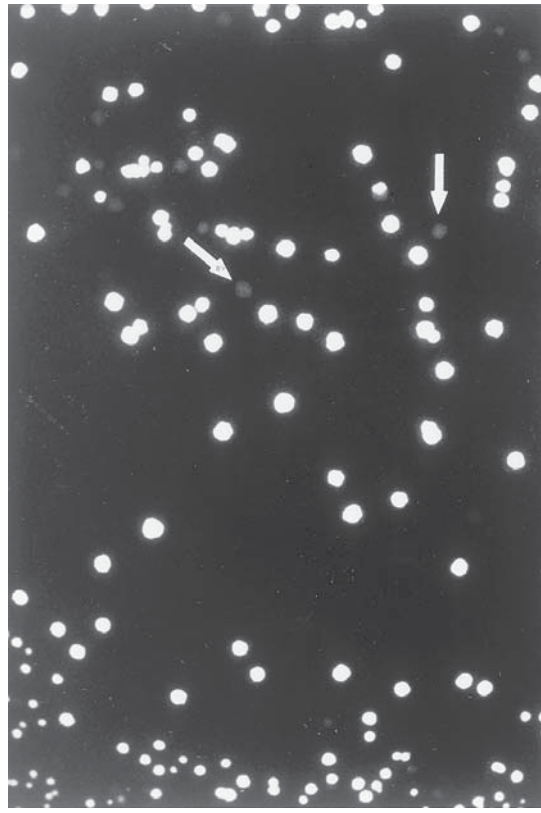


Fig. 4. *E. coli* TB1 that has been transformed with a ligation mixture prepared as described in **Subheading 3.1.** The photograph was taken with a Polaroid camera with an orange filter routinely used for photographing ethidium bromide-stained DNA gels. In black and white photographs, fluorescent colonies are bright white and nonfluorescent colonies are pale gray (*arrows*). (Also on CD-ROM.)

11. The presence of the insert is determined by preparing plasmid DNA from nonfluorescent cells, as described above, and analyzing for presence of the insert on a 1% agarose gel.

3.2. Using Genes Encoding Urogen III Methyltransferase as a Fluorescent Indicator: Overexpression of E. coli cysG^{A} *Gene*

This methodology is based on the use of PCR to amplify all or part of a gene, and, at the same time, provide optimal cloning, transcriptional, and/or translational signals for efficient expression of the gene, either through design of the PCR primers or selection of the vector into which the PCR product is inserted. In the example given, the portion of *cysG* encoding urogen III

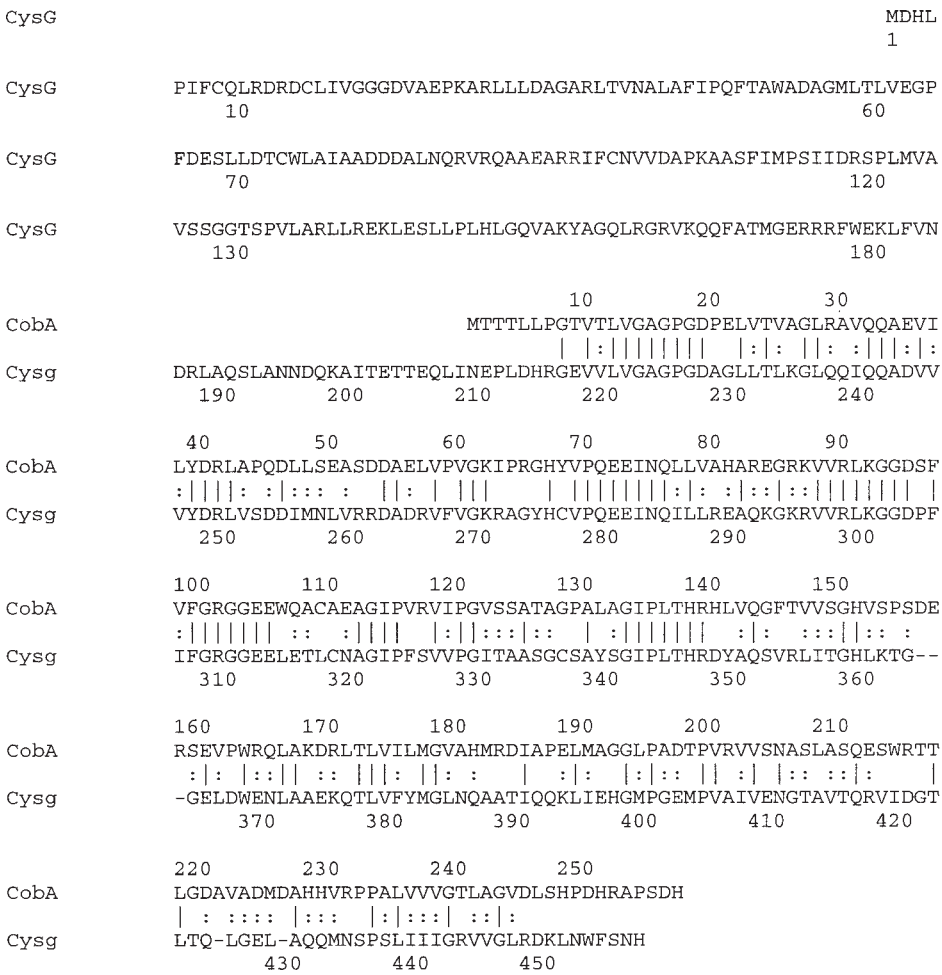


Fig. 5. Homology comparison between CobA and CysG, to determine where urogen III methyltransferase begins in CysG. There is 42.6% identity in a 237-amino acid overlap beginning with amino acid 211 (glutamate) of CysG. (Also on CD-ROM.)

methyltransferase was determined by a homology comparison of CysG with CobA from *P. freudenreichii* using the FASTA program (Genetics Computer Group, Madison, WI) with the result shown in **Fig. 5**. As can be seen, the region of overlap between CysG and CobA begins at Glu 211, and extends almost to the end, so the 5' PCR primer was designed to remove the first 210 codons of *cysG*. The vector chosen for expression of the truncated gene was pUC19, which provides the strong *lac* promoter, but no translational signals. Therefore, a ribosome-binding site, the ATG start codon, and codons for amino

acids 211–220, in addition to a *Bam*HI restriction site (*see* **Note 7**), were incorporated into the 5' primer. The 3' primer was designed to provide the anticodons for the last 10 amino acids of CysG, a stop anticodon, and an *Eco*RI restriction site. Insertion of the PCR product (*cysGA*) into pUC19 results in a plasmid (pEB1), which, when transformed into TB1, affords red fluorescent cells indistinguishable from CR417 ***(3)***.

3.2.1. PCR of Methyltransferase Fragment of cysG^A

1. The following mixture is prepared in a 0.5-mL microcentrifuge tube for PCR amplification of the methyltransferase fragment of the *cysGA* gene: 16 µL water, 5 µL 10X buffer, 8 µL dNTP mix (1.25 m*M*), 5 µL 5' primer (0.01 nmol/µL), 5 µL 3' primer (0.01 nmol/µL), 5 µL $MgCl_2$ (25 mM, *see* **Note 8**), 1 µL pCR252 (100 ng/µL), 1 U *Taq* polymerase, for a total volume of 50 µL.
2. Overlay the mixture with 50 µL mineral oil, and perform 30 cycles of a sequence consisting of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min.
3. At the end of the cycles, remove the mineral oil, and run 5 µL of the PCR mix on a 1% agarose gel.
4. If the product has been synthesized, extract the reaction mix with phenol and chloroform, and ethanol-precipitate the product as described in **Subheading 3.1.3.**
5. Dissolve the dried pellet in 50 µL TE buffer.

3.2.2. Restriction Enzyme Digestion, Ligation, Transformation, and Plating

These procedures are carried out by following all of the steps described above, except that the plasmid is pUC19, the insert is the PCR product, and two restriction enzymes, *Bam*HI and *Eco*RI (or others engineered into the insert by PCR), are used. After plating and an overnight incubation at 37°C, one should be able to observe fluorescent colonies that harbor the recombinant plasmid and express the *cysGA* gene.

4. Notes

1. In the examples given here, the host strain used is TB1 but any strain of *E. coli* that makes urogen III and *S*-adenosyl-L-methionine should work. If the strain overexpresses the *lac* repressor ($lacI^Q$), induction with isopropyl-β-D-thioglactoside may be required.
2. CR417 is available from the author (c-roessner@tamu.edu), and has also been submitted to the Belgian Coordinated Collections of Micro-organisms (http://www.belspo.be/bccm/lmbp.htm).
3. Sources of template DNA for amplification of urogen III methyltransferase genes from other organisms may include plasmids bearing the gene, prokaryotic genomic DNA, or genomic libraries, or cDNA libraries from eukaryotic organisms.
4. The *Bam*HI site, shown in **Fig. 2** apparently was lost during the construction of pISA417, therefore, pISA417 is not cut by *Bam*HI.

5. UV light causes thymidine dimer formation and can result in mutations and cell death. Therefore, exposure of the plates to UV light should be kept to a minimum at all times. Proper eye protection should be used to prevent UV damage to the retina.
6. Addition of ALA (10–20 µg/mL) to the medium may enhance the fluorescence of the colonies bearing nonrecombinant plasmids. However, it may also cause the colonies harboring recombinant plasmids to exhibit faint background fluorescence.
7. Care must be taken that the restriction sites chosen for cloning do not cut within the gene being inserted into the vector.
8. The most critical variable in PCR reactions is the magnesium ion concentration, which should be determined for each set of primers and template. Therefore, a series of concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 m*M*) should be tested. Often, a difference of only 0.05 m*M* will have a drastic effect.

Acknowledgments

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