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High-Level Expression of *Escherichia coli* NADPH-Sulfite Reductase: Requirement for a Cloned *cysG* Plasmid To Overcome Limiting Siroheme Cofactor

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The flavoprotein and hemoprotein components of *Escherichia coli* B NADPH-sulfite reductase are encoded by *cysJ* and *cysI*, respectively. Plasmids containing these two genes overexpressed flavoprotein catalytic activity and apohemoprotein by 13- to 35-fold, but NADPH-sulfite reductase holoenzyme activity was increased only 3-fold. Maximum overexpression of holoenzyme activity was achieved by the inclusion in such plasmids of *Salmonella typhimurium cysG*, which encodes a uroporphyrinogen III methyltransferase required for the synthesis of siroheme, a cofactor for the hemoprotein. Thus, cofactor deficiency, in this case siroheme, can limit overexpression of a cloned enzyme. Catalytically active holoenzyme accounted for 10% of total soluble protein in a host containing cloned *cysJ*, *cysI*, and *cysG*. A 5.3-kb DNA fragment containing *S. typhimurium cysG* was sequenced, and the open reading frame corresponding to *cysG* was identified by subcloning and by identifying plasmid-encoded peptides in maxicells. Comparison with the sequence reported for the *E. coli cysG* region (J. A. Cole, unpublished data; GenBank sequence ECONIRBC) indicates a gene order of *nirB-nirC-cysG* in the cloned *S. typhimurium* fragment. In addition, two open reading frames of unknown identity were found immediately downstream of *cysG*. One of these contains 11 direct repeats of 33 nucleotides each, which correspond to the consensus amino acid sequence Asp-Asp-Val-Thr-Pro-Pro-Asp-Asp-Ser-Gly-Asp.

NADPH-sulfite reductase (SiR) of *Escherichia coli* and *Salmonella typhimurium* catalyzes the reduction of sulfite to sulfide and is required for synthesis of L-cysteine from inorganic sulfate (8, 14). The native enzyme has a subunit structure $\alpha_8\beta_4$, where α_8 is a flavoprotein (SiR-FP) containing both flavin adenine dinucleotide and flavin mononucleotide and β is a hemoprotein (SiR-HP) containing an Fe_4S_4 center and a single molecule of siroheme (24, 25, 37, 39). Electron flow between these cofactors proceeds from NADPH to flavin adenine dinucleotide to flavin mononucleotide in the flavoprotein, then to a closely coupled Fe_4S_4-siroheme center in the hemoprotein, and finally from siroheme to sulfite (38).

The SiR-FP and SiR-HP components of SiR are encoded by *cysJ* and *cysI*, respectively. These genes are contiguous and together with *cysH*, the gene for 3′-phosphoadenosine 5′-phosphosulfate sulfotransferase, comprise an operon with the gene order promoter-*cysJ*-*cysI*-*cysH* (7, 17, 26–29). The *cysJIH* operon is part of the positively regulated cysteine regulon (15) and requires sulfur limitation, CysB protein, and either *O*-acetyl-L-serine or *N*-acetyl-L-serine for expression (10, 11, 14, 28). SiR activity is also dependent on *cysG*, which encodes a uroporphyrinogen III methyltransferase necessary for the synthesis of siroheme (42). This gene is located more than 10 min away from *cysJIH* on the chromosomal map (34) and is not tightly regulated as part of the cysteine regulon (27, 28). In *E. coli*, *cysG* is closely linked to *nirB*, the gene for another siroheme-containing enzyme, nitrite reductase (18). The DNA sequences of *cysG* and the upstream *nirB* and *nirC* genes have been determined for *E. coli* (5a; GenBank sequence ECONIRBC).

Our laboratories are engaged in an effort to characterize the mechanism of electron flow between the Fe_4S_4 cluster and the siroheme moiety of SiR-HP (29). This project involves the generation of specific amino acid substitutions through site-directed mutagenesis of *cysI* and requires 5- to 10-mg quantities of purified mutant proteins for kinetic and spectroscopic analyses. In attempting to construct an overexpressing strain that might facilitate these studies, we have found that siroheme synthesis is limiting for overexpression of SiR-HP enzymic activity from plasmids containing *cysJI*. We describe here how this limitation can be overcome by including *cysG* in such plasmids. We also report the DNA sequence of *S. typhimurium cysG* and the surrounding region.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* JA199 is Δ*trpE5 leu-6 thi hsdR hsdM*$^+$, and EC1124 is a *cysI* derivative of JA199 (28). JM105 was the host for pUC derivatives and for M13 phage propagation (43), and NM522 [*hsd*Δ*5* Δ(*lac-pro*)(F′ *pro*$^+$ *lacI*^{q}*Z*Δ*M15*)] was the host for pT7T3 phagemid derivatives. The *S. typhimurium* strains used were *cysG439* and *cysI68* from the Salmonella Genetic Stock Centre, University of Calgary, Calgary, Alberta, Canada, and LB5000, which is r$^-$ m$^+$ for all three *S. typhimurium* restriction-modification systems (4). LB5000 was the initial recipient for transferring plasmids from *E. coli* by transformation and was made competent by a modification (23) of the method of Hanahan (9). Phage P22HT lysates of LB5000 transformants were then used to transfer plasmids to *cysG439* and *cysI68* by transduction (23).

pRSM10 (Fig. 1) contains *S. typhimurium* LT2 *cysG* on a 5.3-kb fragment from a partial *Sau*3A digest of chromosomal DNA, which was inserted into the *Bam*HI site of pBR322 (23). pJYW2 (not shown) is also a pBR322 derivative con-

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taining the *E. coli* B *cysJIH* region on a 9.5-kb partial *Sau*3A fragment (26, 28, 29). A 5.5-kb *Bam*HI fragment containing *cysJIH* from pJYW2 was subcloned into pBR322 to give pJYW605 (Fig. 1). pJRS102 was constructed by inserting this same fragment into the *Bam*HI site of pRSM10 with the orientation shown in Fig. 1. pJRS101 has the opposite orientation. pJYW609 was constructed by removing a 1.35-kb *Stu*I-*Bal*I fragment from pJYW605 and inserting a 1.9-kb *Pvu*II-*Dra*I fragment from pRSM10 (Fig. 1). Orientation of this fragment in the opposite direction gave pJYW610 (not shown). pJYW609 and pJYW610 do not contain *cysH*.

Double-strength YT (22) was used as the rich medium for growth of JM105 and NM522 and was supplemented with ampicillin (100 μg/ml) and kanamycin (70 μg/ml) for the production of single-stranded pT7T3 DNA in phage M13 capsids (21). Medium E (41) prepared with $MgCl_2$ in place of $MgSO_4$ was our minimal salts medium and was supplemented with 0.5% glucose and either 1.0 mM Na_2SO_4, 0.5 mM L-cystine, 1.0 mM reduced glutathione, or 1.0 mM L-djenkolic acid as a sulfur source. Amino acids at 0.2 mM and thiamine at 4 μg/ml were included where required for auxotrophs.

Recombinant DNA and sequencing methods. Most recombinant DNA methods were those of Maniatis et al. (19) and utilized reagents purchased from Bethesda Research Laboratories, International Biotechnologies, Inc., New England BioLabs, and Pharmacia-LKB Biotechnology Inc. Oligodeoxynucleotides were prepared on an Applied Biosystems model 380A automated DNA synthesizer. DNA sequencing was performed by the method of Sanger et al. (35) with single-stranded templates derived from derivatives of M13 phage or pT7T3 phagemids (21). Overlapping fragments of single-stranded DNA templates were generated from M13 phage derivatives by the method of Dale et al. (6).

Enzyme assays. SiR holoenzyme was assayed as NADPH-hydroxylamine reductase, and SiR-FP was assayed as NADPH-cytochrome *c* reductase (38). Reaction mixtures contained 0.1 M potassium phosphate (pH 7.7), 0.1 mM disodium EDTA, 0.2 mM NADPH, either 10 mM hydroxylamine or 0.1 mM cytochrome *c*, and enzyme in a final volume of 1 ml. Absorbance changes were measured at 25°C in a recording spectrophotometer at 340 nm for NADPH-hydroxylamine reductase and at 550 nm for NADPH-cytochrome *c* reductase. Reaction rates were calculated from an ε_{340} of -6.22 mM^{-1} cm^{-1} for NADPH oxidation and an ε_{550} of 22 mM^{-1} cm^{-1} for cytochrome *c* reduction. By definition, 1 U of SiR holoenzyme activity catalyzes oxidation of 1 μmol of NADPH per min with hydroxylamine as acceptor (two-electron reaction); 1 U of SiR-FP activity catalyzes the reduction of 1 μmol of cytochrome *c* per min (one-electron reaction).

Immunoassay for SiR-HP. SiR-HP apoprotein was measured by an enzyme-linked immunosorbent assay (ELISA) using rabbit antisera to highly purified SiR-HP (37) and horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (HRPO-IgG; Bio-Rad Laboratories). Crude extracts and a standard of purified SiR-HP were diluted with phosphate-buffered saline (PBS; 0.14 M NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4 [pH 7.2]), and antisera were diluted with PBS-T (PBS containing 0.05% Tween-20). Antigens were added in 0.1-ml volumes to 96-well microtiter plates (Immulon-2; Dynatech Laboratories, Inc.) and incubated for 3 h at 37°C or overnight at 4°C. Following removal of the samples by aspiration, the plates were subjected to a wash cycle consisting of three washes with PBS-T and one wash with deionized water. Further nonspecific absorption was blocked by the addition of 0.25 ml of 1% bovine serum albumin in PBS and incubation for 30 min at 37°C. After another wash cycle and air drying, 0.2 ml of a 1/1,000 dilution of anti-SiR-HP was added and incubated for 30 min at 37°C. The plates were again washed, air dried, and incubated for 30 min at 37°C with 0.2 ml of a 1/500 dilution of HRPO-IgG. After another wash cycle and air drying, each well received 0.2 ml of a solution containing 10 mM Na_2HPO_4 (pH 6.0), 0.1 mM disodium EDTA, 1 mg of 5-aminosalicylate per ml, and 0.005% H_2O_2. After 1 to 2 h at 23°C, A_{495} was determined with a microtiter plate reader, and antigen concentrations were calculated from a standard curve.

Enzyme purification. SiR holoenzyme was purified from *S. typhimurium cysI68* containing pJYW609 by the following procedure, which is a significant simplification of the method of Siegel et al. (39) used to purify SiR from wild-type cells. Frozen cells were thawed, suspended in 2 volumes of cold 0.05 M potassium phosphate (pH 7.7)–0.5 mM disodium EDTA (standard buffer), and disrupted by sonic oscillation. Cell debris was removed by centrifugation at 12,000 × *g* for 20 min, and the supernatant was diluted with an equal volume of standard buffer. One-fourth volume of 5% streptomycin sulfate (neutralized with KOH) was added to the supernatant with stirring, and after 10 min the precipitate was removed by centrifugation at 12,000 × *g* for 20 min. SiR holoenzyme was precipitated from the supernatant by the addition of 250 mg of ammonium sulfate per ml and collected by centrifugation. The precipitate was dissolved in a small volume of standard buffer and applied to a column of Superose 6 (1 by 30 cm; Pharmacia-LKB Biotechnology Inc.), equilibrated in standard buffer and run at 0.6 ml/min. SiR holoenzyme was eluted shortly after the void volume. Protein concentrations were determined by the dye-ligand method (2) with bovine serum albumin as a standard. Purified SiR holoenzyme was also quantified by its absorbance with the assumption that ε_{278} = 1.64 ml mg^{-1} cm^{-1} and ε_{386} = 0.46 ml mg^{-1} cm^{-1} (39).

Other techniques. Plasmid-encoded proteins were identified by the maxicell method of Sancar et al. (33). UV-irradiated cells of CSR603 carrying different plasmids were incubated at 37°C for 18 h to promote breakdown of chromosomal DNA and then for 1 h with L-[^{35}S]methionine (1 Ci/mmol; Amersham Corp.). Radiolabeled proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16) and radioautography, using ^{14}C-labeled protein standards from Bethesda Research Laboratories.

RESULTS

SiR expression from cloned *cysJI*. The plasmid-free strain JA199 and strain EC1124 containing pJYW2 (*cysJIH* from *E. coli* B) were grown on minimal medium with either 1 mM reduced glutathione, 1 mM L-djenkolate, 1 mM sulfate, or 0.5 mM L-cystine as a sole sulfur source. Cell extracts were assayed for NADPH-hydroxylamine reductase, which measures SiR holoenzyme activity, and for NADPH-cytochrome *c* reductase, which measures the SiR-FP activity of both the free α_8 flavoprotein and the $\alpha_8\beta_4$ holoenzyme. SiR-FP levels in EC1124(pJYW2) were 13- to 35-fold higher than in comparably grown JA199 and were 20- to 24-fold higher in cells grown on the limiting-sulfur sources glutathione and L-djenkolate than in cells grown on L-cystine (Table 1). The relatively low SiR-FP activity in L-cystine-grown EC1124

TABLE 1. NADPH-sulfite reductase flavoprotein and holoenzyme activities in strains carrying plasmids containing *cysJIH* and *cysG*[a]

Strain	Sulfur source	Activity (U/mg)[b]		Ratio
		NADPH-cytochrome *c* reductase	NADPH-hydroxylamine reductase	
JA199	L-Djenkolate	0.76	0.095	8
JA199	Glutathione	0.95	0.103	9
JA199	Sulfate	0.30	0.027	11
JA199	L-Cystine	0.03	<0.01	
EC1124 carrying:				
pJYW2 (*cysJIH*)	L-Djenkolate	14.8	0.144	103
pJYW2 (*cysJIH*)	Glutathione	12.2	0.184	66
pJYW2 (*cysJIH*)	Sulfate	10.4	0.086	121
pJYW2 (*cysJIH*)	L-Cystine	0.61	0.06	10
EC1124 carrying:				
pJRS102 (*cysJIH cysG*)	L-Djenkolate	3.6	0.39	9
pJRS102 (*cysJIH cysG*)	Glutathione	5.1	0.35	15
pJRS102 (*cysJIH cysG*)	Sulfate	6.1	0.46	13
pJRS102 (*cysJIH cysG*)	L-Cystine	2.4	0.19	13

[a] Cultures were grown with vigorous shaking at 37°C in minimal medium containing 0.5% glucose and either 1 mM L-djenkolate, 1 mM reduced glutathione, mM sulfate, or 0.5 mM L-cystine as the sole sulfur source (14). L-Leucine and L-tryptophan were included at 0.2 mM for JA199 and EC1124. Cells were harvested by centrifugation at densities of 4×10^8 to 6×10^8 cells per ml, and enzyme assays were performed on crude extracts.

[b] NADPH-cytochrome *c* reductase measures SiR-FP activity and is expressed as micromoles of cytochrome *c* reduced per minute. NADPH-hydroxylamine reductase measures NADPH-sulfite reductase holoenzyme activity and is expressed as micromoles of NADPH oxidized per minute.

(pJYW2) was still almost as high as that of sulfur-limited JA199.

In contrast to the large increases in SiR-FP activity, SiR holoenzyme activities in the plasmid strain were increased only threefold over JA199 in sulfate-grown cells and less than twofold in sulfur-limited cells (Table 1). The ratio NADPH-cytochrome *c* reductase NADPH-hydroxylamine reductase in purified SiR holoenzyme is 9.3 (38) and ranged between 8 and 11 in crude extracts of JA199 grown on either L-djenkolate, glutathione, or sulfate. The ratios of 66 to 121 in EC1124(pJYW2) grown on the same sulfur sources suggested that NADPH-hydroxylamine reductase was limited by a relative deficiency of SiR-HP activity. An ELISA assay, however, showed that immunoreactive SiR-HP in sulfur-limited EC1124(pJYW2) was actually 15- to 20-fold higher than in JA199 (data not shown) and equivalent to levels of SiR-FP that were estimated as NADPH-cytochrome *c* reductase. The presence of large amounts of enzymatically inactive SiR-HP in EC1124(pJYW2) suggested the possibility that either the iron sulfide (Fe_4S_4) cofactor or siroheme moieties of this enzyme might be limiting.

We attempted to overcome these putative cofactor deficiencies by adding $FeCl_3$ and the porphyrin percursor δ-aminolevulinic acid (31) to the medium but were unsuccessful. We then considered the possibility that synthesis of siroheme from uroporphyrinogen III might be limiting for holoenzyme activity. This conversion requires two consecutive *S*-adenosylmethionine-mediated transmethylation reactions, which are catalyzed by a single enzyme encoded by *cysG* (1, 42). Plasmids containing both *cysI* and *cysG* were constructed by inserting the *cysJIH* region from *E. coli* B into the *Bam*HI site of pRSM10, a pBR322 derivative that contains the *S. typhimurium cysG* gene (23) (Fig. 1). EC1124 containing one such recombinant, pJRS102, had SiR holoenzyme activities that were 2- to 2.5-fold greater than the highest level obtained with EC1124(pJYW2) and 3- to 4.5-fold greater than the highest level in JA199 (Table 1). Similar results were obtained from pJRS101 (not shown), which differs from pJRS102 only in the orientation of the *cysJIH* insert. These findings indicate that siroheme synthesis is limiting for SiR-HP activity in a strain that overexpresses *cysI*.

DNA sequence of the *cysG* region of *S. typhimurium*. To verify the role of *cysG* in SiR holoenzyme overexpression and to refine plasmid construction, the DNA sequence of the 5.3-kb pRSM10 insert was determined and compared with that reported for this region in *E. coli* (5a; GenBank sequence ECONIRBC). Approximately half of the 5,280-bp insert was sequenced on both strands, including the region between positions 1211 and 2678 (Fig. 2), which contains the open reading frame (ORF) identified as *cysG* (see below). A single strand was sequenced in the regions 1 to 838 and 1098 to 1210 and for about 60% of the region 2678 to 4803. Four complete ORF of greater than 250 codons were found in one direction, and none were found in the other.

In addition to four complete ORF, our sequence begins with a partial ORF of 67 codons, which is followed immedi-

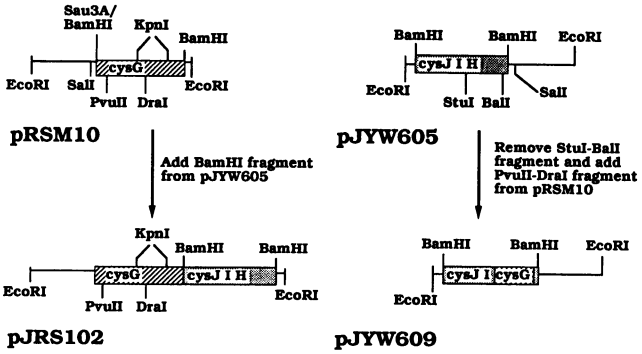

FIG. 1. Plasmids containing *cysJIH* and *cysG*. All are pBR322 derivatives. pRSM10 contains *cysG* from *S. typhimurium* LT2 on a 5.3-kb partial *Sau*3A fragment inserted into the *Bam*HI site. pJYW605 contains *cysJIH* from *E. coli* B on a 5.5-kb *Bam*HI fragment. pJRS102 was constructed by inserting the *cysJIH* fragment from pJYW605 into the single *Bam*HI site of pRSM10. pJYW609 was constructed from pJYW605 by removal of *cysH* as a 1.35-kb *Stu*I-*Bal*I fragment and insertion of a 1.9-kb *Pvu*II-*Dra*I fragment containing *cysG* from pRSM10.

FIG. 2. DNA and deduced amino acid sequences for the pRSM10 insert. The sequence begins with the final 67 codons of an ORF, which may be that of *nirB*. Four additional ORF are shown: ORF-1 corresponds to the *nirC* gene of *E. coli* (5a; GenBank sequence ECONIRBC); ORF-2 is *cysG*; ORF-3 and ORF-4 are of unknown significance. Partial and complete direct repeats are shown in ORF-3. Only the beginning and ending deduced sequence is shown for ORF-4. G+C-rich inverted repeats characteristic of those in rho-independent terminators (32) are shown with a double underline and are present following the putative *nirB* sequence and *cysG*. Shine-Dalgarno (SD) sequences (36) are present at the beginning of ORF-1, *cysG*, and ORF-3. Restriction sites are shown to facilitate comparison with Fig. 3.

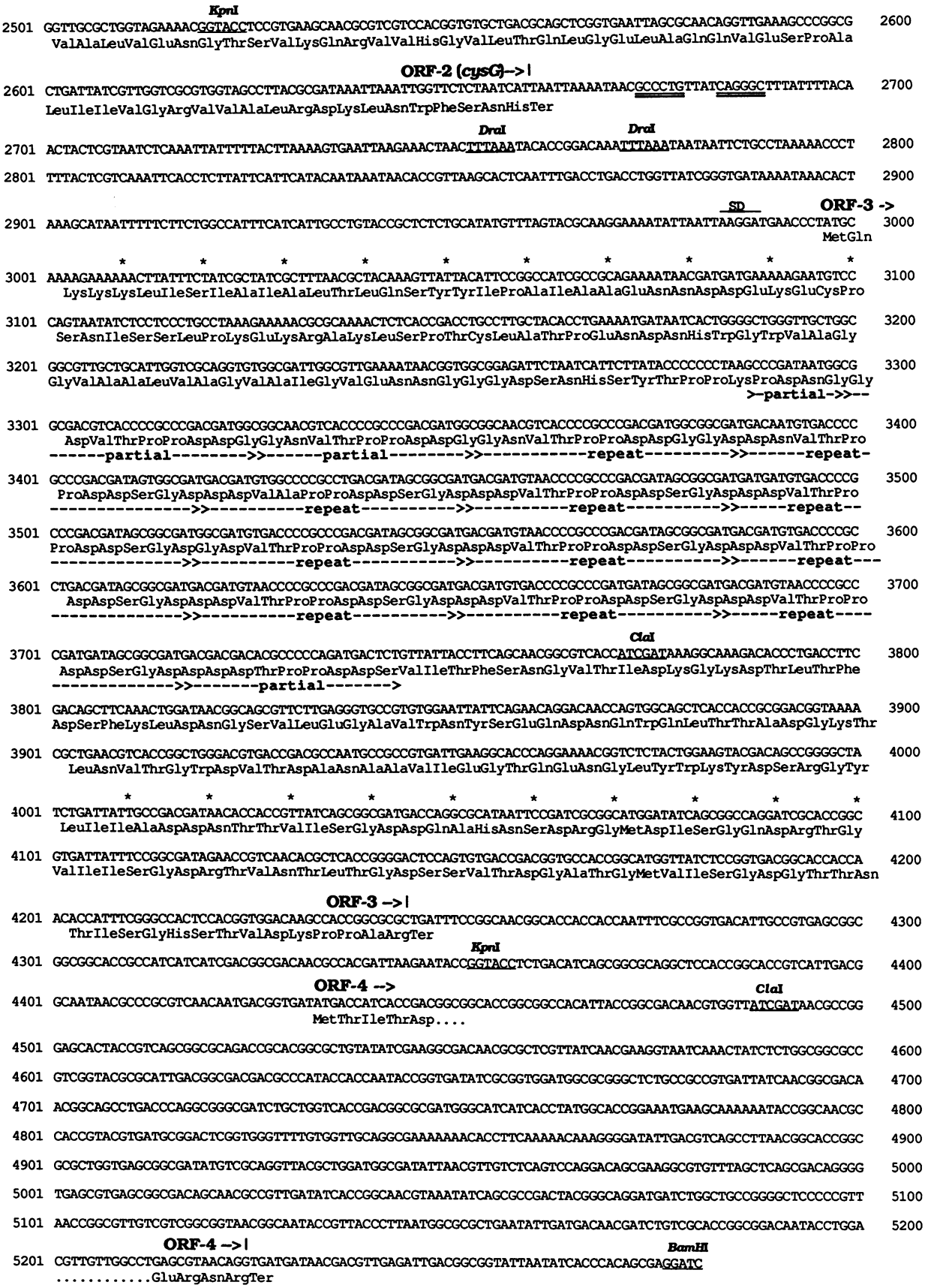

FIG. 2—*Continued.*

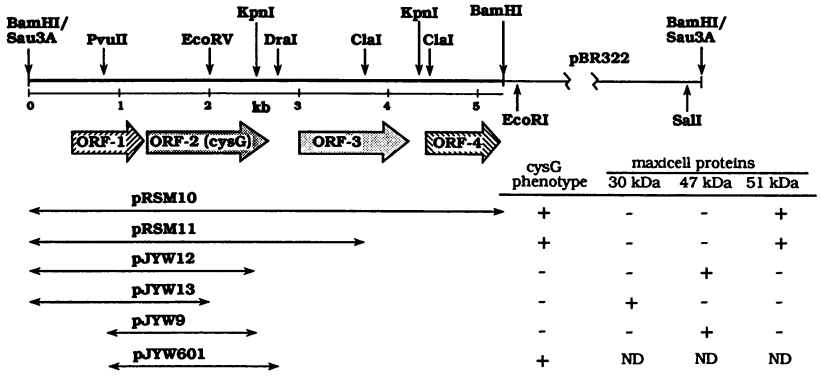

FIG. 3. Functional characterization of pRSM10. Portions of the pRSM10 insert were subcloned in pBR322 and analyzed for their ability to complement *S. typhimurium cysG439* and for expression of plasmid-encoded peptides by the maxicell method (33). Only plasmids containing an intact ORF-2 gave a Cys^+ phenotype with *cysG439* and a 51-kDa peptide in maxicells (see Fig. 4).

ately by a structure resembling a rho-independent terminator (32). This region shows a high degree of identity with the corresponding portion of the *E. coli* sequence, and insertion of 2 additional nucleotides in the *E. coli* sequence gives a comparison with mismatches at 28 of 205 nucleotide positions (not shown) but only three nonsynonymous codons. Beginning at nucleotide position 113, the deduced amino acid sequence, LSDGLCMEDEQ, is very similar to the sequence LSDGLIGDDDN deduced for amino acid residues 984 to 994 of the nitrite reductase of *Aspergillus nidulans* (9a; cited in reference 12). This finding suggests that this partial ORF is the downstream portion of *nirB*, which would extend this gene approximately 340 nucleotides past the termination codon in the reported *E. coli* sequence.

ORF-1 begins with an ATG codon at position 469, which is preceded by an AAGGA Shine-Dalgarno sequence (36), and contains 269 codons corresponding to a hypothetical peptide of 28,545 Da. Comparison with the *E. coli* sequence was again helpful. Insertion of 2 nucleotides in the latter gave a sequence that differed from that of the *S. typhimurium* ORF-1 at 118 nucleotides but contained only 23 nonsynonymous codons; i.e., most nucleotide changes were in the third position of a codon (not shown). The *E. coli* ORF lacks the last codon of the *S. typhimurium* sequence. A portion of this region in the *E. coli* sequence has been designated the putative *nirC* gene, which starts 250 nucleotides after the start codon suggested by our data (5a; GenBank sequence ECONIRBC). We believe our start point to be correct, however, because it is preceded by an excellent Shine-Dalgarno sequence and marks the beginning of a DNA sequence identity that extends for the entire ORF (not shown).

ORF-2 begins at position 1290 with a GTG codon and is also preceded by the Shine-Dalgarno sequence AAGG. It is separated from ORF-1 by only 14 bp and is followed by a rho-independent-like structure (Fig. 2). ORF-2 contains 457 codons corresponding to a hypothetical peptide of 50,057 Da and is very similar to the sequence reported for *E. coli cysG*, which also contains 457 codons (5a; GenBank sequence ECONIRBC). The two deduced peptides differ at 26 codon positions. Additional evidence supporting the identity of ORF-2 as *cysG* is given below.

The 416-codon ORF-3 has two potential ATG start codons, but only the second at position 2997 is preceded by a Shine-Dalgarno sequence. Beginning at codon 120, there are 11 direct full repeats of 33 bp each, which are preceded by three partial repeats of 12, 27, and 27 bp and followed by a partial repeat of 27 bp. The consensus nucleotide sequence for the 11 full repeats is GACGATGTGACCCCGCCCGAC GATAGCGGCGAT, and the consensus deduced amino acid sequence is DDVTPPDDSGD.

ORF-4 begins with an ATG at position 4433 and contains 264 codons specifying a hypothetical peptide of 26,298 Da. Although codon usage is appropriate for an *E. coli* or *S. typhimurium* gene, the absence of a Shine-Dalgarno sequence preceding any ATG or GTG within the ORF suggests that ORF-4 may not be functional.

Identification of *cysG*. Different portions of the pRSM10 insert were subcloned into pBR322 and tested for the presence of *cysG* by their ability to complement *S. typhimurium cysG439* (Fig. 3). Complementation was determined by the presence of a Cys^+ phenotype and occurred only with plasmids containing a complete ORF-2, i.e., pRSM10, pRSM11, and pJYW601. pJYW601 contains a 1.9-kb *Pvu*II-*Dra*I insert, which also includes a small portion of ORF-1. Complementation did not occur with pJYW12 and pJYW13, which contain all of ORF-1 but lack carboxy-terminal portions of ORF-2. The results of these complementation studies were confirmed by analyses of crude extracts from sulfur-limited cells for NADPH-hydroxylamine reductase (data not shown).

We also determined in vivo expression of plasmid-encoded proteins by the maxicell method (33). We found that the $cysG^+$ plasmids, pRSM10 and pRSM11, expressed a 51-kDa peptide, which is close to the 50 kDa expected from ORF-2. This peptide was replaced in pJYW9, pJYW12, and pJYW13 by peptides of 47, 47, and 30 kDa, respectively, which are also close to the sizes predicted from the truncated ORF-2 sequences of these plasmids (Fig. 3 and 4). Bands corresponding to peptides predicted from ORF-1, ORF-3, and ORF-4 were not observed in maxicell experiments. Taken together, these results indicate that ORF-2 is *cysG*.

Purification of SiR from an overexpression strain. SiR holoenzyme was purified from sulfur-limited *S. typhimurium cysI68* containing pJYW609, which carries *cysJ*, *cysI*, *cysG*, and a minimal amount of nonessential insert DNA (Fig. 1). The orientation of *cysG* in this plasmid is downstream of *cysJI* and in the same direction, but it is not known whether this puts *cysG* under control of the *cysJIH* promoter. Similar yields of SiR holoenzyme were obtained from pJYW610, in which *cysG* is oriented toward *cysJI*. *cysI68* carries a deletion in *cysI* and was used to prevent contamination of the *E.*

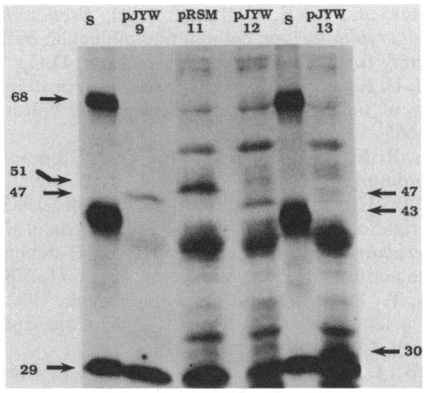

FIG. 4. Expression of plasmid-encoded genes in maxicells. Cultures of CSR603 carrying either pJYW9, pRSM10, pRSM11, pJYW12, or pJYW13 were treated as described by Sancar et al. (33) and radiolabeled with L-[^{35}S]methionine. Plasmid-encoded proteins were identified by SDS-PAGE and radioautography as radiolabelled bands that were not present in the host lacking a plasmid. The four bands of interest are at 47 kDa in pJYW9 and pJYW12, 51 kDa in pRSM11 (and in pRSM10; not shown), and 30 kDa in pJYW13. None of these were present in CSR603 alone or carrying pBR322. The 29-kDa band represents β-lactamase encoded by the pBR322 portion of these plasmids. The two lanes labeled S contain radiolabeled standards of bovine serum albumin (68 kDa), ovalbumin (43 kDa), and carbonic anhydrase (29 kDa).

coli B SiR-HP (encoded by *cysI* on pJYW609) by the product of a chromosomal *cysI* allele. Contamination with *S. typhimurium* SiR-FP is of no consequence to our studies of mutant forms of *E. coli* B SiR-HP but could be eliminated, if necessary, by use of a Δ*cysJI* host. The strain was grown on minimal medium containing 1 mM reduced glutathione as a sulfur source and harvested at a cell density of about 5×10^8/ml. A crude extract from 1.8 g of cell paste (from 2.5 liters of culture) contained 206 U of SiR holoenzyme activity with a specific activity of 1.12 U/mg of protein, which is about 11-fold higher than that obtained from plasmid-free strains of *E. coli* or *S. typhimurium* (14) and represents about 10% of total soluble protein. Purification (described in Materials and Methods) required 1 day and gave 9 mg (100 U) of SiR holoenzyme, which was estimated to be 82% pure by UV-visible light spectral properties (39) and approximately 95% pure by SDS-PAGE. The specific activities of the final product were 11.1 and 346 U/mg for NADPH-hydroxylamine reductase and NADPH-cytochrome *c* reductase, respectively.

DISCUSSION

Failure to overexpress a plasmid-encoded protein may be due to a number of factors, including product insolubility, sensitivity to proteolysis, and toxicity of the product in a given host (3, 20). The studies reported here indicate that the catalytic activity of an overexpressed, cloned gene product can also be limited by deficiency of a specific cofactor. Thus, plasmids containing *cysJ* and *cysI* direct the synthesis of large amounts of SiR-FP catalytic activity and SiR-HP apoprotein, but very little SiR holoenzyme activity because of a lack of the SiR-HP cofactor siroheme. By including *cysG* on such plasmids, we have overcome this limitation and constructed a strain in which SiR holoenzyme accounts for approximately 10% of total soluble protein. This strain should prove useful in future structure-function studies on mutant forms of SiR-HP.

Since an increase in demand for a cofactor will be proportionally greater for one that is rarely used, it is not surprising that siroheme should be so affected, because it is not known to occur in any enzyme other than SiR-HP under aerobic conditions. In contrast, overexpression of SiR-FP activity was not limited by flavin cofactors, which are used by a large number of other enzymes. The increases in SiR holoenzyme activity resulting from adding *cysG* to a *cysJI* plasmid indicates that uroporphyrinogen III methyltransferase, rather than uroporphyrinogen III itself, is the limiting step in siroheme synthesis. This is not unexpected because the amount of uroporphyrinogen III used for siroheme is probably very small compared with that required for total heme synthesis.

Our data are in agreement with previous findings indicating that *cysG* is not tightly regulated as part of the cysteine regulon (27, 28). EC1124(pJYW2) contains only a chromosomal copy of *cysG* yet synthesizes enough siroheme during growth on L-cystine to provide an NADPH-hydroxylamine activity of 0.06 U/mg of protein in a crude extract (Table 1). This level of activity is 60% of that found in the plasmid-free strain JA199 during sulfur limitation (Table 1) and reflects a high capacity for siroheme synthesis under conditions that markedly repress expression of other genes of the cysteine regulon. Siroheme availability may be even higher than estimated in L-cystine-grown cells, since the NADPH-cytochrome *c* reductase/NADPH-hydroxylamine reductase ratio of 10 indicates that siroheme was not limiting. Maximum siroheme synthesis with a single copy of *cysG* can be estimated from EC1124(pJYW2) grown on glutathione, in which siroheme limitation is evident from a NADPH-cytochrome *c* reductase/NADPH-hydroxylamine reductase ratio of 66. The value of 0.184 U of NADPH-hydroxylamine reductase per mg of protein is threefold higher than in L-cystine-grown cells. This difference in apparent siroheme availability could be due to an underestimate in L-cystine-grown cells or may represent some small degree of *cysG* regulation by the cysteine regulon.

The *S. typhimurium* insert of pRSM10 contains three complete ORF in addition to ORF-2, which was identified as *cysG* by genetic complementation and maxicell experiments and by comparison with the *E. coli* sequence. The first portion of our sequence also appears to contain the downstream end of *nirB*, as judged by homology of the deduced amino acid sequence with that of the carboxyl-terminal portion of *A. nidulans* nitrite reductase (12). Comparison with the *E. coli* sequence indicates that ORF-1 is *nirC*, the function of which is unknown (5). The sequence of genes in our cloned sequence is then *nirB–nirC–cysG*–ORF-3–ORF-4. The genetic organization of this region suggests that *nirC* may be coexpressed with *cysG* rather than with *nirB*, since *nirB* is followed by a structure that appears to be a rho-independent terminator, and *nirC* and *cysG* are separated by only 11 bp. Furthermore, using primer extension techniques, we have been unable to locate an in vivo transcription start site in the 500 bp upstream of *cysG*.

The function, if any, of ORF-3 and ORF-4 is unknown. ORF-3 is remarkable for the presence of 11 repeats of 33 nucleotides each. The pattern of nucleotide variation within these repeats suggests that they encode a functional peptide. For instance, only 6 of 18 nonconsensus nucleotides give nonsynonymous codons, and 5 of these result in conservative amino acid changes, e.g., Asp → Asn and Asp → Gly. The consensus deduced peptide for these repeats contains

Asp at 5 of the 11 positions, and the predicted net change for the hypothetical peptide for the entire ORF-3 is −77. Our failure to detect such a peptide in maxicell experiments does not rule out the possibility of expression from ORF-3, since such a highly negative charge might be expected to cause anomalously slow migration in SDS-PAGE (13, 30, 40).

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