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NOTES

Cloning and Expression of a Structural Gene from *Chlorobium vibrioforme* That Complements the *hemA* Mutation in *Escherichia coli*

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***Escherichia coli* SASX41B carries the *hemA* mutation and requires δ-aminolevulinic acid for growth. Strain SASX41B was transformed to prototrophy with pYA1, a plasmid vector carrying a 5.8-kilobase insert of genomic DNA from the green sulfur bacterium *Chlorobium vibrioforme*. Cell extracts prepared from transformed cells are able to catalyze transfer of label from [1-^{14}C]glutamate or [3,4-^{3}H]glutamyl-tRNA to δ-aminolevulinic acid at rates much higher than extracts of wild-type cells can, whereas extracts prepared from untransformed strain SASX41B cells lack both activities. By comparing the relative abilities of glutamyl-tRNAs derived from several heterologous cell types to function as substrates for the dehydrogenase reaction in extracts of HB101 and SASX41B cells transformed by pYA1, it was determined that the expressed dehydrogenase in the transformed cells resembled that of *C. vibrioforme* and not that of *E. coli*. Thus it can be concluded that plasmid pYA1 contains inserted DNA that codes for a structural component of *C. vibrioforme* glutamyl-tRNA dehydrogenase which confers glutamyl-tRNA substrate specificity.**

The *hemA* mutant strain SASX41B of *Escherichia coli* K-12 was first described by Săsărman et al. in 1968 (28, 29). This mutant is an auxotroph requiring the heme precursor δ-aminolevulinic acid (ALA) for growth on minimal media. The *hemA* locus has been mapped at 27 min on the *E. coli* genetic linkage map (5, 13, 28, 29). *E. coli* forms ALA from glutamate by the five-carbon pathway (3, 17, 25), which was first characterized in plants and algae (6, 11, 22, 35). In this pathway, the α-carboxyl group of glutamate is first activated by ligation to $tRNA^{Glu}$. The activated glutamate is next reduced to glutamate-1-semialdehyde or a closely related compound (15). The glutamate-1-semialdehyde is then converted to ALA through an aminotransferase reaction (2, 14). Although originally thought to be restricted to plants and algae, the five-carbon ALA biosynthetic pathway has recently been found to occur in many other species of bacteria (4, 10, 23, 24, 26).

It was previously assumed that *E. coli* forms ALA via condensation of glycine and succinyl-coenzyme A, which is catalyzed by ALA synthase, the reaction that is used by purple nonsulfur bacteria, fungi, and animal cells. When the ALA-requiring *hemA* strain SASX41B was first described (28, 29), it was generally presumed to be defective in ALA synthase activity. The ability of cloned DNA from *Rhizobium meliloti* (16), *Bradyrhizobium japonicum* (12), and *Rhodobacter sphaeroides* (17, 33) and mouse cDNA (32) to transform SASX41B cells to ALA independence when present in the cells on a multicopy plasmid was interpreted as supporting the assumption that *E. coli* normally uses the ALA synthase reaction to form ALA. This assumption seemed reasonable because all of the donor species form ALA via the ALA synthase reaction. Also, an *E. coli* strain that apparently lacks succinyl-coenzyme A synthetase was reported to be unable to grow anaerobically unless supplemented with ALA along with methionine and lysine (20). Although ALA formation was detected in crude extracts of untransformed wild-type *E. coli* cells when the cells were incubated with succinyl-coenzyme A and glycine, rigorous determination of substrate requirements and reaction mechanism was not reported (33).

More recently, it was determined that strain SASX41B forms ALA from glutamate when the strain is transformed by a multicopy plasmid carrying genomic DNA from *hem*$^{+}$ *E. coli* (17). The sequence of the *E. coli hemA* gene was determined independently in several laboratories (7, 18, 34). The sequence has no similarity to any published ALA synthase gene sequence. Two genes, named *hemA* and *hemL*, complementing two different ALA auxotrophs of *Salmonella typhimurium*, have also been cloned. The function of these genes is still unknown, but the sequence of the *hemA* gene shows remarkable homology with that of the *E. coli hemA* gene (8, 9). Finally, it was found that the common laboratory strain of *E. coli*, HB101, forms ALA exclusively via the five-carbon pathway, even in the absence of a plasmid (3). Moreover, it was established by in vitro analysis and enzyme complementation experiments that the enzyme that is lacking in strain SASX41B is glutamyl-tRNA dehydrogenase (3). Thus strain SASX41B appeared to be usable as a host for selecting a cloned gene required for glutamyl-tRNA dehydrogenase activity.

The *hemA* gene cloned from *E. coli* and *S. typhimurium* may encode a structural component of the enzyme glutamyl-tRNA dehydrogenase. Alternatively, *hemA* may encode some regulatory function that is needed for expression of the dehydrogenase gene. We have cloned DNA from *Chlorobium vibrioforme*, a green sulfur bacterium, that complements the *hemA* mutation in *E. coli*.

On the basis of several earlier results, *C. vibrioforme* was

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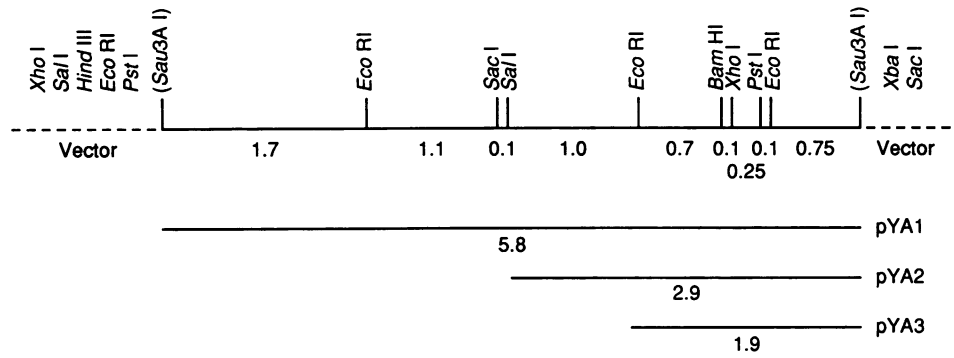

FIG. 1. Restriction map of the 5.8-kb *C. vibrioforme* genomic DNA fragment in plasmid pYA1 and subclones pYA2 and pYA3. The restriction enzymes used were *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, *Sac*I, *Sal*I, *Xba*I, and *Xho*I. There is a single restriction site for each of these enzymes in the polylinker region of the pBluescript vector. The original *Bam*HI cloning site is lost upon ligation and replaced by a *Sau*3AI site.

chosen as a suitable donor species for obtaining transforming DNA capable of complementing SASX41B cells. First, it has been established that *C. vibrioforme* forms ALA exclusively by the five-carbon pathway (4, 27). Second, because *C. vibrioforme* is a procaryotic species, its genomic DNA would be more likely to be expressed in *E. coli* than would that of a eucaryotic plant or an algal species. Third, because *C. vibrioforme* forms large amounts of tetrapyrroles for use as photosynthetic pigments, there was a possibility that its genes that encode steps of ALA formation would be expressed at much higher levels than those of *E. coli*, which forms relatively small amounts of tetrapyrroles. Fourth, unlike many of the in vitro enzyme systems derived from plants, algae, and cyanobacteria, *C. vibrioforme* extracts were able to use *E. coli* $tRNA^{Glu}$ to support ALA formation. Thus there was a reasonable expectation that if the *C. vibrioforme* gene for glutamyl-tRNA dehydrogenase could be expressed in *E. coli*, the gene product would function with *E. coli* glutamyl-tRNA in vivo and thereby confer prototrophy on the *hemA* cells. Fifth, tRNAs from several heterologous cell types were examined for their relative abilities to support ALA formation in extracts of *C. vibrioforme* (27). It was hoped that if extracts of transformed *E. coli* cells had a spectrum of acceptable tRNAs that was similar to that of *C. vibrioforme* extracts and different from that of *hem*$^+$ *E. coli* extracts, it could be established that the cloned DNA encodes a structural component of the glutamyl-tRNA dehydrogenase enzyme that confers tRNA substrate specificity rather than a regulatory gene required for the expression of *E. coli* glutamyl-tRNA dehydrogenase.

E. coli ALA auxotrophic strain SASX41B carrying the *hemA* mutation was obtained from B. J. Bachmann of the *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, Conn. *E. coli* HB101 (*hem*$^+$) was obtained from W. Tapprich, Brown University, Providence, R.I. *E. coli* HB101 containing plasmid pBR322 was obtained from M. Dolan, Brown University, and *E. coli* DH5α was from A. Laras, Brown University. Strains HB101 and DH5α were grown in LB medium (19). Strain SASX41B was grown in M9CA medium (19) supplemented with 20 μM ALA. HB101(pBR322) and SASX41B(pYA1) cells were grown on LB or M9CA medium supplemented with ampicillin (AMP; 100 μg/ml). Cultures were grown in Erlenmeyer flasks on a rotary shaker (200 rpm) at 37°C. *Chlorella vulgaris* Beijerinck strain C-10, *Euglena gracilis* Klebs var. Pringsheim wild-type strain Z and the derived aplastidic strain W_{14}ZNalL, *C. vibrioforme* f. *thiosulfatophilum* NCIB 8327, and *Synechocystis* sp. strain PCC 6803 were grown and used for the preparation of cell extracts and tRNA, as described previously (1, 21, 26, 27). The pBluescript SK(+) phagemid vector was obtained from Stratagene (La Jolla, Calif.); restriction enzymes and T4 DNA ligase were from Pharmacia LKB Biotechnology (Piscataway, N.J.) and New England BioLabs, Inc., (Beverly, Mass.); DNA size markers were from BRL-Life Technologies (Gaithersburg, Md.); *E. coli* tRNA mixture and pancreatic RNase A were from Sigma Chemical Co. (St. Louis, Mo.); [3,4-^{3}H]glutamate and [1-^{14}C]glutamate were from Du Pont-New England Nuclear (Boston, Mass.); and all other reagents were from Sigma, Fisher Scientific Co. (Medford, Mass.), or Research Organics (Cleveland, Ohio).

Genomic DNA (0.6 mg) from *C. vibrioforme* f. *thiosulfatophilum* NCIB 8327 (a generous gift from J. G. Ormerod, University of Oslo, Oslo, Norway) was partially digested with 8 U of *Sau*3AI in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA [pH 7.5]) for 20 min at 37°C, and fragments in the size range of 5 to 6 kilobases were purified by sucrose gradient centrifugation (19). The pBluescript vector (10 μg in 50 μl of TE buffer) was completely digested by incubation with 125 U of *Bam*HI. Ligation was carried out in a mixture containing 100 ng of DNA and 80 U of T4 DNA ligase in 10 μl of TE buffer, with a vector-to-insert molar ratio of 1:3.

Because strain SASX41B, the intended selection host, is not restriction negative, it was necessary to pass recombinant plasmids containing *C. vibrioforme* DNA through *E. coli* DH5α (*hsdR1* [r_K^- m_K^+]), which lacks a restriction system but retains the ability to methylate DNA and can thus transform the foreign DNA replicating in the cell into a suitably methylated form. Strain DH5α (Dam$^+$ Ddcm$^+$) can methylate both adenine and cytosine. Furthermore, because strain DH5α lacks a functional *lacZ* gene, it was possible to determine the fraction of plasmids containing inserts within the *lacZ* gene by scoring blue and white colonies on plates containing 100 μg of AMP per ml, 50 μM isopropyl-β-D-thiogalactoside, and 0.8 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Competent DH5α cells (2.5 ml) were transformed with 0.5 ml of ligation mixture. Plasmid DNA was isolated by the boiling method of Maniatis et al. (19).

The isolated plasmid DNA was used to transform SASX41B cells. Transformants were plated on M9CA agar plates supplemented with AMP (100 μg/ml) with or without ALA. From a total of 1.5×10^6 transformants plated on medium without ALA (estimated by colony formation on medium with ALA), a single colony capable of growing on medium without ALA was recovered and analyzed. The transformed cells grew on solid M9CA medium containing

TABLE 1. ALA formation from glutamate by extracts of HB101, SASX41B, and SASX41B(pYA1) *E. coli* cells[a]

Cell extract source		Preincubation mixture	ALA formation (cpm/mg of protein)
Strain	Phenotype		
HB101	Hem^+	Buffer	1,020
HB101	Hem^+	RNase	20
SASX41B	HemA	Buffer	10
SASX41B	HemA	RNase	10
SASX41B(pYA1)	Hem^+	Buffer	9,230
SASX41B(pYA1)	Hem^+	RNase	40

[a] Samples contained 100 μl of gel-filtered supernatant from high-speed centrifugation (500 to 800 μg of protein) from extracts of HB101 (*hem*$^+$), SASX41B (*hemA*), or SASX41B(pYA1) cell extract. All samples were preincubated at 37°C for 20 min with or without 0.25 μg of RNase A as indicated. Incubation was started by the addition of 1 μCi of [1-^{14}C]glutamate in 50 μl of a substrate-cofactor mixture, resulting in a final concentration of 10 μM glutamate, 2.0 A_{260} units of *E. coli* tRNA mixture, and the ingredients listed in the text. The net counts per minute tabulated are the values after subtraction of the background of 40 cpm obtained when the substrate was incubated in the reaction mixture in the absence of cell extract.

AMP and lacking ALA at a rate comparable to that of *hem*$^+$ HB101 cells growing on a similar medium in the absence of the antibiotic.

The plasmid (pYA1) contained in the transformed cells was isolated and found to contain a 5.8-kilobase insert (Fig. 1). The fact that the Hem^+ phenotype is due to the presence of the pYA1 plasmid was confirmed by the transformation of competent SASX41B cells with the plasmid isolated from cells derived from the initial colony. All of the cells transformed by this plasmid acquired AMP resistance and ALA independence simultaneously. The insert in pYA1 was mapped by restriction analysis. Subclone pYA2 was obtained by complete digestion with *Sal*I and contains a 2.9-kilobase insert. Subclone pYA3 was obtained by partial digestion with *Eco*RI and contains a 1.9-kilobase insert. Both pYA2 and pYA3 are able to transform SASX41B cells to ALA independence.

Cell extracts of *E. coli* cultures for enzyme assays were obtained and partially purified as previously described (3). Assays of ALA formation from glutamate were carried out in 1.5-ml microcentrifuge tubes for 1 h at 37°C following (when indicated) a 15-min preincubation of the extract in the presence or absence of 0.25 μg of RNase A. The reaction volume (250 μl) contained 50 to 200 μl of extract in assay buffer and 50 μM glutamate (added after preincubation and containing 10 μCi of [3,4-^{3}H]glutamate or 1 μCi of [1-^{14}C] glutamate), 1 mM NADPH, 5 mM ATP, 5 mM levulinate, and 20 μM pyridoxal-P. ALA was isolated and its radioactivity was measured as previously described (1, 3, 4).

Cell extracts prepared from SASX41B(pYA1) cells formed ALA from glutamate at rates far exceeding those of HB101 cells, whereas extracts of untransformed SASX41B cells formed no ALA (Table 1).

The extracts derived from HB101(pBR322) and SASX41B (pYA1) cells were examined for their relative abilities to use, as substrates for ALA formation, glutamyl-tRNAs derived from *E. coli* and from several species and strains of bacteria and algae. tRNAs from *Chlorella*, *Euglena*, *Chlorobium*, and *Synechocystis* species were prepared and purified by phenol-chloroform extraction and DEAE-cellulose chromatography as previously described (30). The [3,4-^{3}H]glutamyl-tRNA was prepared enzymatically by the method of Schneegurt et al. (31). In each case, the cell extract used for charging the tRNA was from the same source (cell type or species) as the RNA. In this experiment, the relative amounts of protein

TABLE 2. ALA formation from glutamyl-tRNA by extracts of HB101(pBR322) and SASX41B(pYA1) *E. coli* cells[a]

Cell extract source		ALA formation	
For tRNA and Glu-tRNA formation	For ALA formation	Net cpm	% *E. coli*
E. coli HB101	HB101(pBR322)	8,970	100
	SASX41B(pYA1)	20,800	100
C. vibrioforme	HB101(pBR322)	111	1
	SASX41B(pYA1)	3,910	19
Synechocystis sp. (cyanobacterium)	HB101(pBR322)	460	5
	SASX41B(pYA1)	7,130	34
Chlorella vulgaris (green alga)	HB101(pBR322)	80	1
	SASX41B(pYA1)	5,130	25
Euglena gracilis Z (wild type)	HB101(pBR322)	190	2
	SASX41B(pYA1)	5,520	27
Euglena gracilis W_{14}ZNalL (aplastidic)	HB101(pBR322)	0	0
	SASX41B(pYA1)	0	0

[a] The total amount of radioactivity in the various glutamyl-tRNAs used was 85,600 to 98,800 cpm. Incubations were started by the addition of 25 μl of assay buffer containing freshly dissolved labeled substrate. Incubations contained (in 250 μl of assay buffer) 150 μl of *E. coli* HB101(pBR322) extract (1.35 mg of protein) or 25 μl of *E. coli* SASX41B(pYA1) extract (0.17 mg of protein) and the ingredients listed in the text. The net counts per minute tabulated are the values after subtraction of the background of 390 cpm obtained when the substrate was incubated in the reaction mixture in the absence of cell extract.

from HB101(pBR322) and SASX41B(pYA1) cells that were added to the incubations were adjusted to minimize the difference in ALA-forming activity that occurs with *E. coli* glutamyl-tRNA as the substrate. To control for any possible influence by a plasmid in the transformed SASX41B(pYA1) cells, HB101(pBR322) cells were used instead of HB101 cells as a source of Hem^+ extract, and both strains were grown in medium containing 100 μg of AMP per ml. It was determined that the presence of pBR322 does not measurably influence the ALA-forming activity of HB101 cells (data not shown). The incubation mixtures contained SASX41B(pYA1) or HB101(pBR322) cell extract in assay buffer, [3,4-^{3}H]glutamyl-tRNA prepared as described above, 1 mM glutamate, 5 mM K-levulinate, 1 mM NADPH, and 20 mM pyridoxal-P. Incubation was for 40 min at 37°C. ALA was isolated and its radioactivity was measured as previously described (1, 3, 4).

Extracts from HB101(pBR322) cells formed appreciable amounts of ALA only from *E. coli* glutamyl-tRNA, and label from none of the heterologous glutamyl-tRNAs was incorporated into ALA at a level greater than 5% of that from *E. coli* glutamyl-tRNA (Table 2). In contrast, extracts from SASX41B(pYA1) cells used all of the heterologous substrates except that derived from aplastidic *Euglena* strain W_{14}ZNalL, with label incorporation ranging from 19 to 34% of that from *E. coli* glutamyl-tRNA. The relative abilities of the glutamyl-tRNAs derived from heterologous sources to serve as substrates for ALA formation in the extracts of SASX41B(pYA1) cells closely matched the relative abilities of tRNAs from these sources to support ALA formation from glutamate in extracts of *C. vibrioforme* (27). In *C. vibrioforme* extracts, all of the tested RNAs, with the exception of RNA obtained from extracts of aplastidic *Euglena* cells, could support ALA formation at rates, per unit of added RNA, of 50 to 300% of the rate with *C. vibrioforme* RNA (27). Because the substrate acceptability of the enzyme in transformed cells resembled that of the DNA donor species, it can be concluded that the cloned *C. vibrioforme* DNA in plasmid pYA1 encodes the glutamyl-tRNA dehydrogenase enzyme or a structural component of it that confers glutamyl-tRNA substrate specificity.

In conclusion, the cloning and identification of a DNA fragment from the phototrophic green sulfur bacterium *C. vibrioforme* that encodes a structural component of glutamyl-tRNA dehydrogenase will aid in ongoing studies of the structure of this enzyme, its catalytic mechanism, the regulation of its expression, and its role in regulating tetrapyrrole biosynthesis via the five-carbon pathway.

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