(1)

細菌におけるδ-アミノレブリン酸生合成に関する研究

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緒　　言

δ－アミノレブリン酸(ALA)は、ヘム、コリン、クロロフィル、チトクロム等、テトラピロール化合物の最初の前駆体であり、生物に普遍的に存在する重要なアミノ酸である。(1)

さらに近年、ALAに安全性の高い感光性除草剤としての用途が見いだされ注目されている。現在ALAは化学合成法により生産、市販されているが非常に高価であり、安価な合成法が開発されれば除草剤をはじめとする広範な用途開発が進展するものと期待されている。微生物によるALA生産も種々検討されているが未だ工業的生産には至っておらず、ALA高生産株の育種が必要とされている。

現在までALA生合成の研究は主として呼吸系酵素－ヘムの合成－の微生物学観点から行われてきた。酵母を好気培養した時には高等動植物と同様なチトクロム系を示すのに対し、嫌気状態で培養した時では不完全なチトクロム系しか示さないことが1930年頃Warburgらにより指摘され(2,3)、その後ミトコンドリアの構造、電子伝達系やTCAサイクルなどの呼吸酵素系の生成と調節の機構が詳しく解析されてきた(4,5,6,7,8)。そしてヘム合成の最初の中間体がALAであり、ALA synthaseにより合成されること、ALA synthaseはヘム合成の律速段階でありヘムにより抑制されることが明かとなった(9)。現在、酵母においてALA synthaseをコードするHEM1遺伝子はcytochrome oxidase subunit 4(COX4)遺伝子と同様にHAP2-HAP3により調節されていることが明かとなっている(10)。一方、細菌においてはミトコンドリアは存在しないことから、呼吸酵素系生成の調節といっても、酵母の場合とは大幅に異なり、また細菌間における呼吸様式の多様性から考えてみても酵素系生成の調節に関しても多面的であることが考えられる。1957年にBeljanskiらはStaphylococcus aureusからヘム無添加の培地中で増殖できない変異株を単離した(11)。これらの変異株はチトクロム、カタラーゼ等いわゆる呼吸鎖系の合成酵素が欠損しており、これらの形態はリーキーに似た、増殖が遅い不明瞭なコロニー　”SCV(Small colony variant)またはNcf⁻(Normal colony formatin-deficient)と呼ばれる”を形成した。これらの変異株はその後詳しく解析された。大腸菌ではその変異はゲノム中に広く存在しておりhemC、hemD、hemGの遺伝子はオペロンを形成していることが分かってきた(12,13)。hemB(14,15)、hemC(16)、hemD(13,17)、hemG(18) 遺伝子はクローニングされ、ヒト(hemB,hemC)、ラット(hemB)、酵母(hemB)から単離された遺伝子と高いホモロジーを示し、ヘム合成経路が多くの種に渡って保存されていることが示された。このようなヘムに至る合成経路を、FIG.1.1に示す。

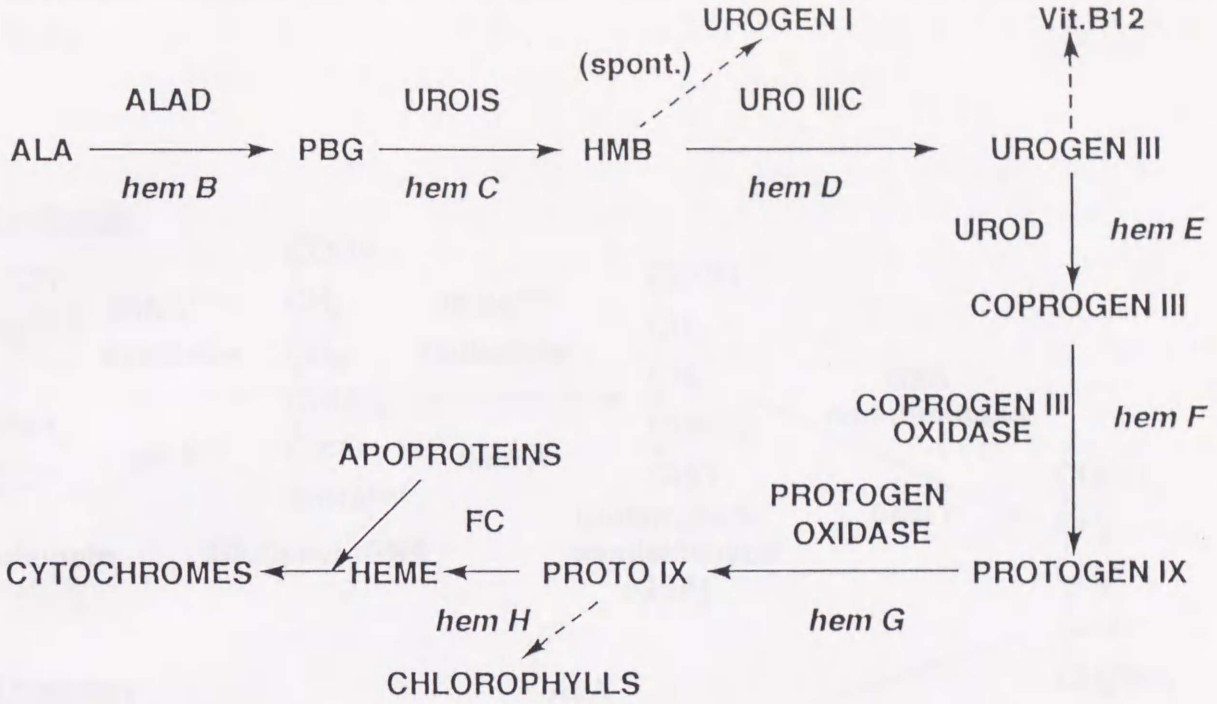

FIG. 1. 1 Simplified scheme of the biosynthesis of heme.
ALA, δ-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethylbilane; UROGEN I and III , uroporphyrinogen I and III; Vit .B12, vitamine B_{12}; COPROGEN III, coproporphyrinogen III; PROTOGEN IX, protoporphyrinogen IX; PROTO IX, protoporphyrin IX; ALAD, δ-aminolevulinic acid dehydratase; UROIS, uroporphyrinogen-I synthase (porphobilinogen deaminase); UROIIIC, uroporphyrinogen-III cosynthase; UROD, uroporphyrinogen-III decarboxylase; COPROGEN III OXID, coproporphyrinogen-III oxidase; FC, ferrochelatase.

このヘム合成の最初の前駆体であるALAの合成には、２つの経路の存在が報告されている。最初に発見されたのがグリシンとスクシニルCoAからALA synthase(ALAS)(succinyl-CoA: glycine C-succinyl transferase [decarboxylating], EC 2.3.1.37)によって合成されるShemin(C4)経路である。この経路は、動物、かび、酵母、光合成細菌等で確認されている(19)。もう１つの経路はグルタミン酸の炭素骨格がそのままの形で保存されるC5経路である(20)。この経路は植物、藻類で見いだされ、３つのステップからなるとされている。第一のステップは、グルタミン酸がglutamyl-tRNA synthaseにより触媒されglutamyl-tRNAとなる反応で、これはタンパク質合成と共役している。次にglutamyl-tRNAはNADPH-dependent reductase (glutamyl-tRNA reductase [EC5.4.3.1])によってglutamate-1-semialdehyde(GSA)もしくはその環状体である2-hydroxy-3-aminotetrahydropyran-1-oneへと変換される。GSAはさらにGSA aminomutase によりアミノ基の分子内転移が起こりALAが合成される。この経路は当初植物、藻類にお

いてのみ存在するとされてきたがEscherichia coli(21) やSalmonella typhimulium(22)等の細菌においてもその存在が確認された。この2つの経路をFIG.1.2に示す。

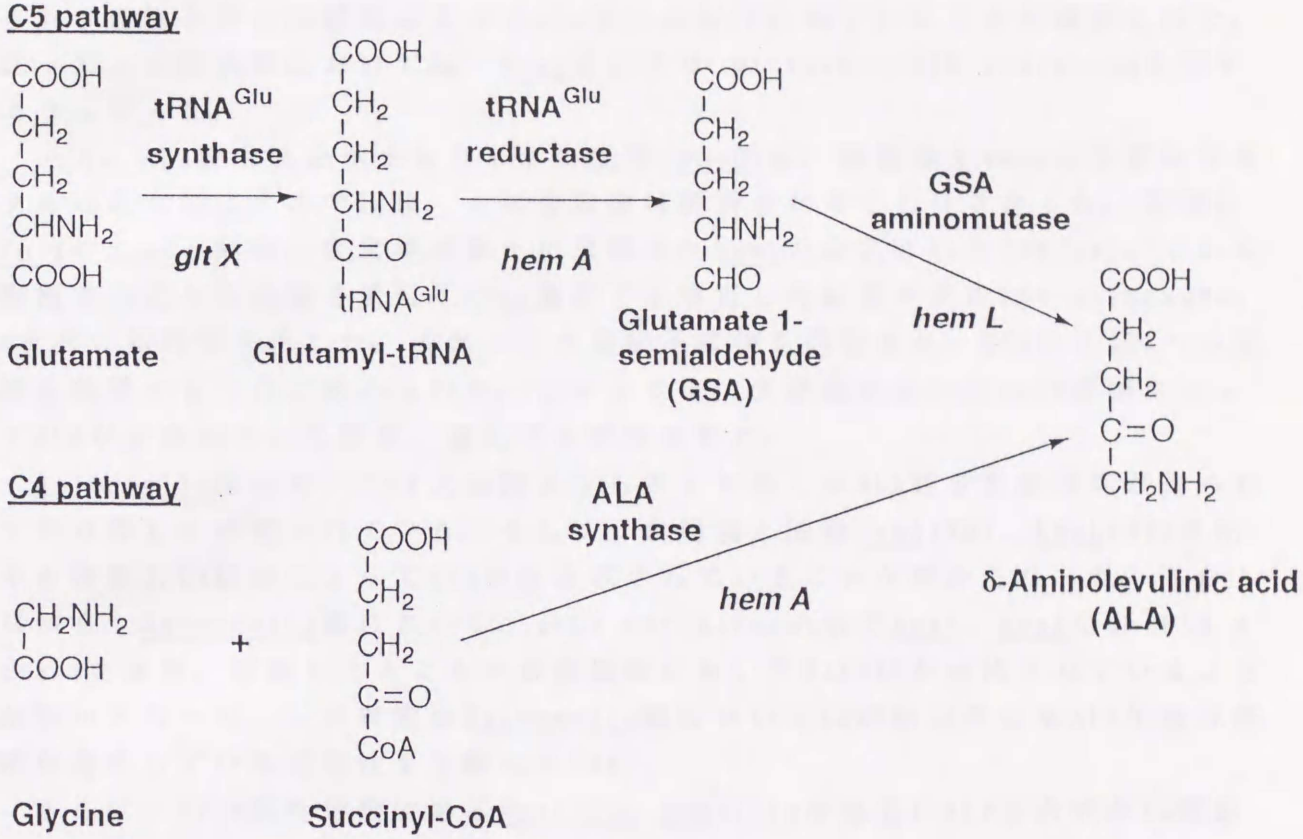

FIG. 1.2 Simplified scheme of the biosynthesis of δ-aminolevulinic acid

大腸菌のALA生合成経路の研究は、1960年代にE.coli K-12から数種のALA要求性変異株が単離されたことに始まる(23,24,25)。これらの変異はすべてALA無添加の培地で増殖できない要求性変異株である。これらの変異の1つはhemAと呼ばれ、染色体上の位置は27minであった。もう1つの変異はpopCと呼ばれ4minに存在していた。当時大腸菌においてもALA合成は唯一ALA synthaseによって生合成されると考えられていたので、この変異はALA synthase欠損によるものとされ、hemAと命名された。このことは1958年Kikuchiらの大腸菌からALASを精製し大腸菌におけるALA合成はC4経路よるものであるという報告(26)や、他の種のALA synthase遺伝子を大腸菌に導入した形質転換体は、ALA無添加の培地中でも増殖できるという実験事実によっても支持されていた。

1989年、3つのグループにより大腸菌hemA遺伝子が単離されその構造等が解

析された(27,28,29)。さらに、Salmonella typhimurium(30)、Bacillus subtilis(31)においてもhemA遺伝子が単離され、これら遺伝子間には高い相同性が見られた。しかしながら、既に単離されている動物や光合成細菌のhemA(ALA synthase)遺伝子とは相同性は認められなかった。このhemA遺伝子が単離された年に、1960年代に単離されたALA要求性変異株を材料として大腸菌のALA生合成経路が明らかにされた(32)。その結果、ALA synthase(C4,Shemine)活性は認められず植物と同じC5経路によってALA生合成が行われていることが確認された。従って、大腸菌等においては、hemA遺伝子はglutamyl-tRNA reductaseを指すようになった。

一方、ALA要求性を示すもう1つの変異(popC)は、染色体上4minの位置に存在することが報告されて以来、この変異株は解析されることはなかった。近年E.coliのpopCと同様の変異株が新たに単離されhemLと命名された(33,34)。これを相補することの出来る遺伝子hemL遺伝子を解析した結果大麦のGSA aminomutaseと高い相同性を示した。また、この遺伝子産物も同定され、GSAからALAへの変換を触媒することが認められた。このように、大腸菌においてはC5経路によってALAが生合成され各酵素、遺伝子も同定された。

Salmonella菌においては大腸菌よりもさらに多くのALA要求性変異株が得られており詳しく研究されている。そして、大腸菌と同様hemA(30)、hemL(35)遺伝子も存在しC5経路によってALAが生合成されていることが明かとなった。しかしながら、Salmonella菌において、gene replacement法でhemA、hemLのdouble mutantを単離、解析したところその変異株においてVitB12が合成されていることが明かとなった。この事実はSalmonella菌においてC5経路以外にもALA生合成経路が存在している可能性を示唆した(36)。

さらに、Gram陽性細菌であるBacillus subtilisは過去にALA生合成がC4経路によって行われていることが報告されていたが、近年大腸菌やSalmonellaと同様にC5経路によってALAが生合成されていることが確認された。また染色体上での位置は前者ではhemA、hemL遺伝子は染色体上に散在しているのに対し、Bacillusにおいてはhem AXBCDLオペロンを形成していることが明かとなった(37)。

このように、細菌においてはALAの生合成はC5経路がmajorな経路として同定されているが別の経路の可能性を示唆する結果も得られており、より詳細な解析が求められている。そこで、本研究においてEscherichia coliを材料としてALA生合成機構の解析を行った。

一方ALAの応用面において、イリノイ大学のRebeizらがALAの散布が雑草（双子葉植物等）に対し殺草効果のあることを見いだした(38)。雑草の多くが双子葉植物に分類されることから、選択性除草剤としての活用を提案しており、自然界での分解性も高いことから、新しいタイプの除草剤として注目されている。さらにRebeizらは、ALAがキャベツしゃくとり虫等の有害虫に対しても殺虫性があることを報告している(39)。

植物に吸収されたALAはテトラピロール化合物の一種であるPchlid（プロトクロロフィリド）に変換され、光化学反応によって活性酸素が生じ、これが植物の

リポプロテインを酸化し、枯死に至るとされている。この反応は”光力学的除草”と呼ばれ、主として双子葉植物で発現し、単子葉植物では起こりにくいとされている(38)。ALAによる除草効果は3-5mMの比較的低濃度で有効であり、直接、雑草や葉の茎に噴霧すれば2-3日で100%に近い殺草効果があると報告されている(38)。

ALAは現在、化学合成法によって生産され市販されているが、非常に高価であり価格は1g当り数千円から1万円もする。従って、除草剤として大量に使用するにはより安価な製造法の開発が望まれている。そこで微生物、特に高いALAの生産能力がある光合成細菌を用いての発酵生産の研究が行われている。

このように、δ-アミノレブリン酸はテトラピロール化合物の最初の前駆体として重要であるばかりでなく、農薬としても注目を集めているアミノ酸である。

本研究においては、Escherichia coliのALA生合成機構とその遺伝子レベルでの発現調節機構の解明と、植物病原菌でxanthan gum などの有用多糖生産菌であるXanthomonas campestris pv. phaseoli、ビタミンB_{12}生産菌であるPropionobacterium freudenreichiiの細菌を材料として、ALAの大量生産を目的とした。

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第 1 章

Cloning and characterization of genes involved in the biosynthesis of δ-aminolevulinic acid in *Escherichia coli*.

INTRODUCTION

The synthesis of ALA is the first step in the biosynthesis of hemes, chlorophylls, corrins, and bile pigments (11). The biosynthetic pathway from ALA to hemes involves seven different enzymatic steps and seems to be highly conserved in animals, plants, and bacteria. ALA is synthesized by either of two major pathways. In the C4 pathway (7), ALA is formed by condensation of glycine and succinyl CoA. This reaction is catalyzed by ALA synthase (EC 2.3.1.37), which is found in animal cells, yeasts, fungi, and certain bacteria. In the C5 pathway, ALA is formed from glutamate by a series of reactions which include activation of glutamate by ligation to tRNA, reduction of the activated glutamate to yield GSA by an NAD(P)H-dependent reductase, and transamination of GSA to form ALA by a GSA 2,1-aminotransferase (EC 5.4.3.8) (Fig. 1). The C5 pathway is found in plant chloroplasts, cyanobacteria, anaerobic archaebacteria, E. coli, and Salmonella typhimurium. It has been reported that genes complementing an ALA-auxotrophic HemA$^-$ mutant of E. coli encode a structural component of glutamyl tRNA dehydrogenase (3, 10, 17). Complementation of the ALA-auxotrophic HemL$^-$ mutations in E. coli (6) and S. typhimurium (4) yielded genes that encode GSA aminotransferase. However, little is known about the molecular basis of the metabolic regulation of the synthesis of ALA.

In this report, we describe the isolation of two classes of ALA-requiring mutants, both of which are defective in the C5 pathway for synthesis of ALA. A study of the genes that complemented these mutants led us to a new gene that appeared to be involved in the synthesis of ALA. We also showed some evidence that

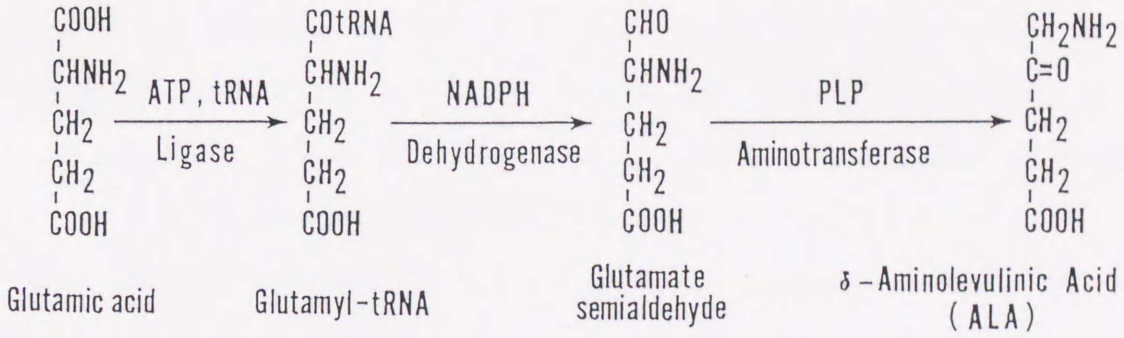

Fig. 1. The C5 pathway for formation of ALA. PLP is the pyridoxal phosphate.

E. coli may have an alternative, minor pathway for the ALA synthesis where *hemA* is involved.

RESULTS

(a) Isolation and characterization of mutants deficient in the biosynthesis of ALA

Mutants requiring ALA for growth (Ala^-) were isolated from strain C600 by mutagenesis with NTG. Six Ala^- mutants (I14, I21, I23, I24, I33, and I45) were obtained (Table I). None of these Ala^- mutants grew even in LB medium unless it was supplemented with ALA. These mutants required ALA at more than 25 μg/ml for maximum growth. These mutants had strictly non-leaky phenotypes unlike the Hem^- mutants isolated by others (14, 12).

The defect of ALA formation from glutamate in Ala^- mutants was examined. A cell extract of the parental strain C600 was capable of generating labeled ALA from 1-[^{14}C]glutamate but not from 2-[^{14}C]glycine (Table II) as reported previously (1). In contrast to the extract of Ala^+ cells, the cell extracts of all the Ala^- mutants were unable to generate labeled ALA from 1-[^{14}C]glutamate. These results indicate that all the Ala^- mutants isolated were defective in the formation of ALA via the C5 pathway from glutamate.

(b) Classification of Ala^- mutants by the cloned genes

To determine the defective loci of Ala^- mutants, the genes that allowed recovery of growth of Ala^- mutants in medium without ALA were cloned from the chromosomal DNA of *E. coli* C600. Fragments from the chromosomal DNA partially digested by EcoRI were ligated with EcoRI-cleaved pBR322 and introduced into competent cells of strain I21. After a three-day incubation at 37°C, 16 Ala^+ clones which grew up on the selective plate (LB agar supplemented with 100 μg Ap/ml without ALA) were isolated. All the

TABLE I

List of *E. coli* strains[a]

Strain	Genotype	Source or reference
E. coli C600	*hsdR hsdM thr*-1 *leu*-6 *thi*-1 *hem*$^+$	Ogawa, H.
I14	*hemM*14	This study
I21	*hemL*21	This study
I23	*hemL*23	This study
I24	*hemM*24	This study
I33	*hemM*33	This study
I45	*hemL*45	This study
JM109	*relA supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi* Δ(*lac-proAB*)[F'/*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15] *mcrA*$^-$ *mcrB*$^+$	Yanisch-Perron et al. (18)

[a]Mutagenesis with NTG (25 μg/ml) and 50 μg Cm/ml was achieved as described by Sklar (15). The mutagenized cells were spread on plates of LB (1% tryptone [Difco]/ 0.5% yeast extract / 0.5% NaCl) that contained ALA (50 μg/ml), and the colonies were replicated on synthetic medium (0.5% glucose/ 0.2% ammonium sulfate/ 0.05% KH_2PO_4/ 0.4% Na_2HPO_4/ 0.01% $MgCl_2.6H_2O$/ 0.001% each of NaCl, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$ and $CaCl_2.2H_2O$). Amino acids (25 μg/ml) and vitamins (1 μg/ml) were also added to the synthetic medium.

TABLE II

Formation of ALA from glutamate by extracts of parent and mutant cells

Source of cell extract[a]	Incubation mixture	Labeled substrate	Incubation time (min)	ALA formation cpm/mg protein[b]
C600	Complete	[^{14}C]Glu	0	50
	Complete	[^{14}C]Glu	90	750
	Complete	[^{14}C]Gly	0	<40
	Complete	[^{14}C]Gly	90	<40
I14	Complete	[^{14}C]Glu	0	<40
	Complete	[^{14}C]Glu	90	60
	Complete	[^{14}C]Gly	90	<40
I21	Complete	[^{14}C]Glu	90	<40
I23	Complete	[^{14}C]Glu	90	<40
I24	Complete	[^{14}C]Glu	90	<40
I33	Complete	[^{14}C]Glu	90	<40
I45	Complete	[^{14}C]Glu	90	<40

[a]**Methods**: Cell extracts were prepared as described by Avissar and Beale (1). Cultures of E. coli C600 were grown for 16 h at 37 °C in LB medium. Cells were washed twice with extraction buffer (0.1 M Tris pH 7.9/ 0.3 M glycerol/ 15 mM $MgCl_2$/ 3 mM DTT). The cell pellet was suspended in 4 ml of extraction buffer per g of cells. The suspension was sonicated at 4°C and centrifuged at 10,000 x

g for 10 min. Low-molecular-weight materials were removed from the supernatant by passage through a column of Sephadex G-25 equilibrated with assay buffer (0.1 M Tris pH 7.9/ 1 M glycerol/ 15 mM $MgCl_2$/ 1 mM DTT). The extract was used directly or stored at -80°C until used for enzyme assays.

[b]**Methods**: Assays of ALA synthesis were performed as described by Avissar and Beale (1). For the C5 pathway, the 50-µl reaction mixture was composed of the desired amount of cell extract, 50 µM glutamate that included L-1-[^{14}C]-glutamate (NEN Research Products)/ 1 mM NADPH/ 5 mM ATP/ 5 mM levulinate/ 20 µM pyridoxal phosphate/ 0.1 OD_{260} units of glutamyl tRNA from E. coli (Boehringer Mannheim Biochem.). Reactions were incubated at 37° C for 90 min. For the C-4 pathway, the reaction was performed as described by Kikuchi et al. (7). Incubation was terminated by the addition of 20 µl of 0.5 M Na.phosphate buffer pH6.8/ 10 µl of acetylacetone/ 5 µl of 1 mM ALA. The resulting mixture was boiled for 20 min and cooled rapidly to room temperature. The denatured protein was removed by centrifugation. ALA pyrrole in the supernatant was purified by ion-exchange chromatography or direct chromatography on Whatmann 3MM paper with the upper layer of a mixture 10 N ammonia:n-butanol:H_2O (1:49:50, v/v) as solvent (16). The spots of radioactive ALA pyrrole on the paper chromatography were cut out and radioactivity was assayed in a liquid scintillation counter. In the colorimetric assay, ALA was quantitated spectrophotometrically at 553 nm after reaction with acetylacetone and Ehrich reagent (16).

plasmid prepared from each transformant was confirmed to have the ability to retransform strain I21 to Ala^+. Interestingly, the transformants exhibited heterogeneous growth on the selective plate. A smaller number of transformants grew up as variously sized colonies, presumably because their growth started after various length lag periods. This phenomenon was observed only for the growth of transformants on the selective plates, just after the uptake of DNA. Once these transformants grew, they continued to grow normally.

Restriction maps of the isolated DNA fragments were constructed (Fig. 2A). All plasmids carried an identical 18-kb EcoRI-EcoRI insert (pAS002) with exception of pAS001 (Fig. 2A). Plasmid pAS001 lacked a large part of the insert that included one of the EcoRI junctions between the insert and the vector. Deletion analysis showed that the gene complementing the Ala^- mutation was located within a 1.4-kb HpaI-EcoRI fragment (Fig. 2A). The pAS023 plasmid that contains only this fragment was used to transform variety of Ala^- mutants. pAS023 also complemented Ala^- strains I23 and I45, as well as strain I21. These transformants also exhibited the heterogeneous growth. Other Ala^- mutations (I14, I24 and I33), by contrast, were not complemented by pAS023 at all. From these results, we classified the mutant strains into at least two groups that corresponded to mutations at different loci. Thus, we tentatively designated the former class of mutations as $AlaA^-$.

(c) Determination of the gene deficient in the $AlaA^-$ mutation

The map position on the E. coli chromosome was determined

for the gene that complemented AlaA$^-$. A physical map of the *E. coli* chromosome generated from overlapping lambda phage clones that encompassed the entire genome of *E. coli* was used (8). As a probe for the gene complemented AlaA$^-$, the 1.4-kb *Hpa*I-*Eco*RI fragment from pAS023 (Fig. 2A) was isolated and labeled with a non-radioactive DNA-labeling kit (Boehringer Mannheim, F. R. G.). The probe hybridized to the coordinate position of clone 15A7, 9H2 and 4E4 on the gene mapping membrane (Takara Shuzo Co. Ltd., Kyoto, Japan). This result indicated that the gene complementing the AlaA$^-$ mutation was located at 4 min on the genome.

The nt sequences of both strands of the 1.4-kb *Hpa*I-*Eco*RI fragment of pAS023 were determined by the dideoxy chain-termination method (13). A homology search using GENETYX programs (SDC Software Development Co., Ltd., Tokyo, Japan) revealed that the gene essential for complementing AlaA$^-$ was identical to *hemL*, the gene that encodes GSA aminotransferase, which has been mapped at 4 min and sequenced previously (6). Although our isolated *hemL* lacked the 245 bp at the 3'-end that correspond to 82 aa residues at the C-terminus of the HemL protein, it complemented the mutations in I21, I23 and I45. The complete *hemL* gene isolated from the Kohara clone 15A7 was also found to strictly complement the AlaA$^-$ mutation but not others. From these results, we concluded that the AlaA$^-$ mutants were defective in the locus known as *hemL*.

(d) Cloning and identification of genes that complemented AlaB$^-$

To investigate the defective loci of other mutants than AlaA$^-$ (*hemL*), the genes to recover their growth in the selective plate were cloned by using pBR322 into the strain I33 as a host.

One positive clone was obtained. The resultant plasmid pAS332 that contained a 11-kb EcoRI-EcoRI fragment complemented Ala^- mutant strains I14 and I24 as well as strain I33 (Fig. 2B), suggesting that these three mutants belong to the same class of Ala^- mutations, which was tentatively designated $AlaB^-$. Thus, our Ala^- mutants were genetically classified into two groups with mutations at different loci, $AlaA^-$ (hemL) and $AlaB^-$. The latter mutants seemed to have a defect in glutamyl tRNA dehydrogenase in the C5 pathway for synthesis of ALA.

Plasmid pAS332 was introduced into the $AlaA^-$ mutants, I23, I21, and I45. Unexpectedly, pAS332 complemented the $AlaA^-$ mutation too and the transformants formed small colonies on the selective plates. The weakness in the growth was also observed in the successive plate culture and the liquid culture. This phenomenon is clearly different from the leaky phenotype of mutation. This result indicates that plasmid pAS332 should carry more than one gene involved in ALA synthesis. Thus, further deletion analysis was carried out to determine the region essential for complementation of the Ala^- mutation. The $AlaB^-$ mutation was also complemented by the deletion plasmids pAS334, pAS335 and pAS336, as well as by pAS332 (Fig. 2B). However, the mode of complementation changed to heterogeneous, as observed in the case of the transformation of $AlaA^-$ mutants with pAS023. No complementation of the $AlaA^-$ mutation was observed with plasmids pAS334, pAS336, or pAS337. This result suggests that there is an additional gene that was lost during construction of deletion plasmids from pAS332. As expected, another deletion plasmid, pAS333

(Fig. 2B), complemented both AlaA$^-$ (*hemL*) and AlaB$^-$ mutations and exhibited small colonies. Furthermore, we subcloned the 2.3-kb *Pst*I-*Nhe*I fragment on pAS333 into a low-copy-number plasmid, pRK290, which possesses a P1 type replicon and generates only a few copies per host chromosome (2). The same mode of complementation by this plasmid was also observed, indicating that these phenomena were not caused by only the high-copy-number plasmid.

(e) Characterization of the genes that complemented AlaB$^-$ and its relation with *hemA*

To localize the genes complemented AlaB$^-$ and all Ala$^-$ mutations, the 3.6-kb *Pst*I-*Eco*RI fragment from pAS332 was used as a probe. This probe hybridized to the coordinate position of clones 12A3, 4D10, 13H6 on the gene mapping membrane (8). This result shows that the gene complementing AlaB$^-$ or both AlaA$^-$ and AlaB$^-$ are located at 27 min on the chromosome where *hemA* is known to be located (14).

Fig. 3 shows the nt sequence of the 2.0-kb *Sal*I-*Nae*I fragment from pAS332. The nt of the gene that complemented AlaB$^-$ can be read clockwise on the chromosome and consists of 621 bp, with a putative ATG start codon at nt position 360 and a TAA termination codon at nt position 981 (Fig. 3). The putative start codon is preceded by a nt with a high degree of similarity to the -10 and -35 consensus sequences and includes a potential ribosome-binding site (AGGA). The gene product was deduced to be a 23-kDa polypeptide consisting of 207 aa residues. This protein has no homology with either the known ALA-associated proteins or any other protein that is known to date. This protein appeared to be a novel enzyme that catalyzes a reaction in the synthesis of ALA in the

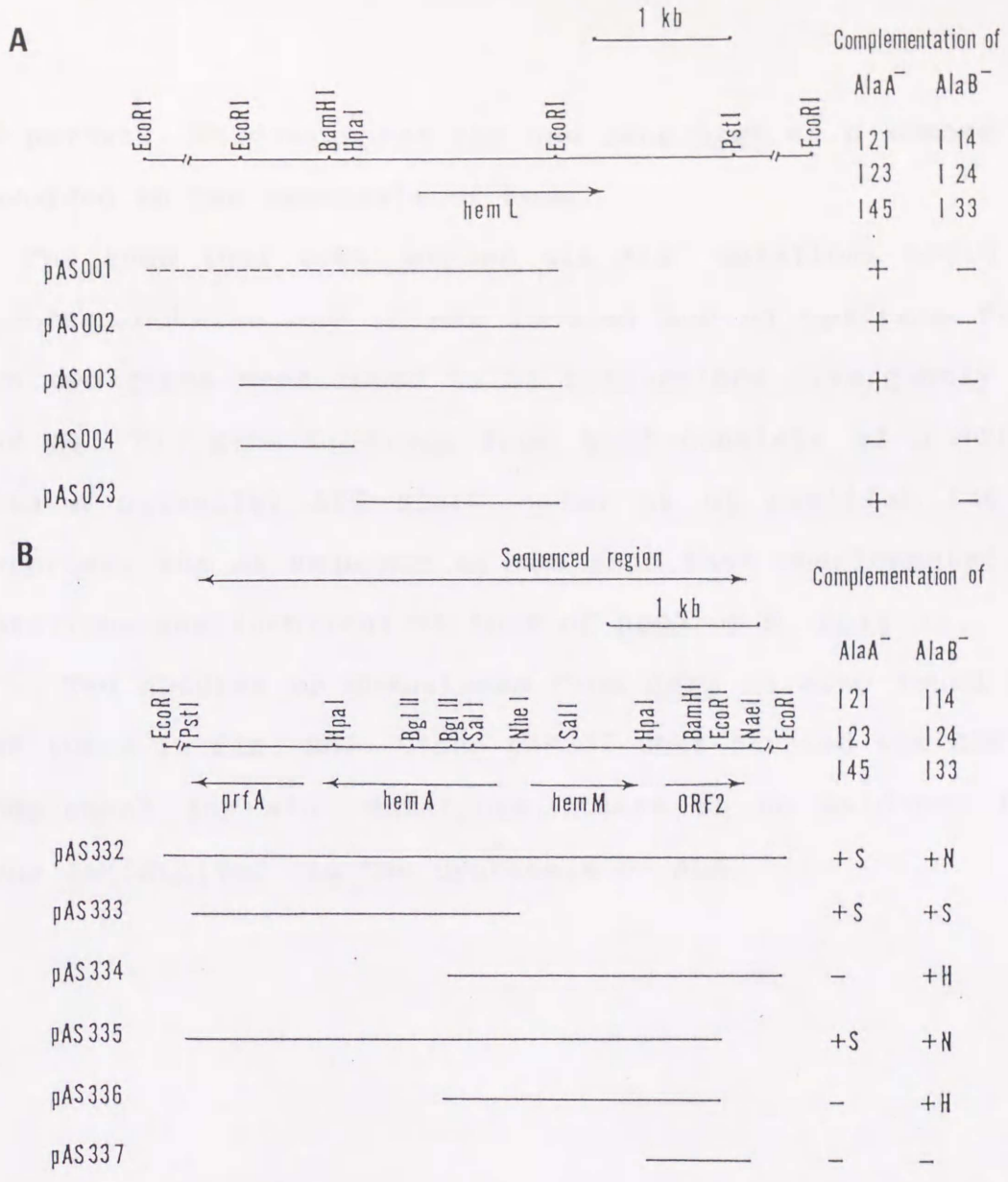

Fig. 2. Deletion maps of the hemL region (A) and the hemA and hemM region (B) and complementation test of AlaA⁻ and AlaB⁻ mutations. The top horizontal line represents the E. coli chromosome. The broken line of pAS001 indicates spontaneous deletion occurred during transformation. The lines of plasmids indicate remaining DNA fragments. The directions of transcription are indicated with arrows. Symbol + in map A represents the complementation but observed heterologous (mixed with small and normal) colonies. The letters of S, H, and N in map B indicate small, heterologous, and normal colony formations, respectively. Methods: Plasmids prepared by a mini-scale preparation were used to transform mutant strains (I14, I21, I23, I24, I33, and I45) and the complemented colonies grew on the selective plate (LB agar with 100 μg Ap/ml without ALA) were observed.

C5 pathway. We designated the new gene hemM as a member of genes involved in the synthesis of heme.

The gene that complemented all Ala$^-$ mutations could be read counterclockwise and it was located 200 bp upstream from hemM. The two genes were found to be transcribed divergently (Figs. 2 and 3). The gene upstream from hemM consists of a 1254-bp ORF with a potential ATG start codon at nt position 146. To our surprise, the nt sequence of the gene that complemented all Ala$^-$ mutations was identical to that of hemA of E. coli.

Two hundred bp downstream from hemM we also found a 609-bp ORF (ORF2 in Fig. 2B). Since pAS337 that carried the ORF2 did not complement any Ala$^-$ mutations, there is no evidence that this gene is involved in the synthesis of ALA.

```
  1 SalI
     Ser Leu Val Val Gly Gly Gln Val Met Pro Gln Ala Leu Leu Ser Asp Leu Ala Gln
5'-GT CGA CAG CAC CAC GCC GCC CTG CAC CAT CGG CTG CGC AAG CAG GCT GTC AAG CGC CTG
  60
  Asp Leu Lys Asp Pro Ser Phe Ser Val Arg Glu Arg Leu Ser Val Pro Ala Thr Lys His
  ATC GAG CTT ATC CGG CGA AAA CGA TAC ACG TTC TCG CAG CGA TAC AGG TGC CGT TTT ATG
  120                          ←— hemA
  Asn Ile Gly Leu Ala Leu Leu Thr Met
  GTT GAT ACC GAG TGC TAA AAG GGT CAT  GTCTGCGGGAAATAATACCAACGTTGATAGGGTTAGTCTGCT
  189
  TGCATCATACAGGATGCGTAAGATCAATAAAAGAGAGCGCCCCCTTTTGGAGTAATTGCCGAAAGCCGTTAGATTCTGG
  268                                        NheI                              SD
  CAATTAAGACAACTTGAACATAGACGATAGCGGACGGTAACGCTAGCATTAAGGGTTATAACTGCAACGTATCTCAAGG
  347              hemM —→
  ACTTGTCATCACT   ATG CCC CTG CCC GAT TTT CGT CTT ATC CGC CTC GTA CCG CTG GCT GCT
                  Met Pro Leu Pro Asp Phe Arg Leu Ile Arg Leu Val Pro Leu Ala Ala
  408               1                                                          16
  CTT GTG CTC ACT GCC TGT TCC GTT ACC ACG CCC AAA GGT CCT GGC AAA AGC CCG GAT TCG
  Leu Val Leu Thr Ala Cys Ser Val Thr Thr Pro Lys Gly Pro Gly Lys Ser Pro Asp Ser
  468                                                                        36
  CCA CAA TGG CGT CAG CAT CAG CAA GAC GTG CGC AAT CTT AAT CAG TAT CAG ACT CGC GGC
  Pro Gln Trp Arg Gln His Gln Gln Asp Val Arg Asn Leu Asn Gln Tyr Gln Thr Arg Gly
  528                                                                        56
  GCG TTC GCT TAT ATT TCT GAC CAA CAA AAA GTG TAC GCC CGC TTT TTC TGG CAG CAA ACC
  Ala Phe Ala Tyr Ile Ser Asp Gln Gln Lys Val Tyr Ala Arg Phe Phe Trp Gln Gln Thr
  588                                                                        76
  GGC CAG GAT CGC TAC CGT CTG CTG CTC ACT AAC CCA TTG GGC AGC ACG GAA CTG GAG CTG
  Gly Gln Asp Arg Tyr Arg Leu Leu Leu Thr Asn Pro Leu Gly Ser Thr Glu Leu Glu Leu
  648                                  SalI                                  96
  AAT GCT CAA CCG GGT AAC GTG CAG TTA GTC GAC AAT AAA GGT CAG CGT TAT ACC GCC GAT
  Asn Ala Gln Pro Gly Asn Val Gln Leu Val Asp Asn Lys Gly Gln Arg Tyr Thr Ala Asp
  708                                                                       116
  GAC GCC GAA GAG ATG ATT GGC AAA TTG ACC GGA ATG CCA ATT CCG CTC AAC AGC TTG CGC
  Asp Ala Glu Glu Met Ile Gly Lys Leu Thr Gly Met Pro Ile Pro Leu Asn Ser Leu Arg
  768                                                                       136
  CAG TGG ATT TTA GGT TTA CCG GGT GAT GCA ACC GAC TAC AAA CTG GAC GAC CAG TAC CGC
  Gln Trp Ile Leu Gly Leu Pro Gly Asp Ala Thr Asp Tyr Lys leu Asp Asp Gln Tyr Arg
  828                                                                       156
  CTG AGC GAA ATT ACC TAC AGC CAG AAT GGC AAA AAC TGG AAG GTT GTT TAT GGT GGT TAT
  Leu Ser Glu Ile Thr Tyr Ser Gln Asn Gly Lys Asn Trp Lys Val Val Tyr Gly Gly Tyr
  888                                                                       176
  GAC ACC AAA ACG CAA CCT GCG ATG CCA GCC AAT ATG GAA CTC ACC GAC GGT GGT CAA CGC
  Asp Thr Lys Thr Gln Pro Ala Met Pro Ala Asn Met Glu Leu Thr Asp Gly Gly Gln Arg
  948                                                                       196
  ATC AAG TTA AAA ATG GAT AAC TGG ATA GTG AAA TAA  TGCGGACACAGTGGCCCTCTCCGGCAAAAC
  Ile Lys Leu Lys Met Asp Asn Trp Ile Val Lys ***
  1014                                207
  TTAATCTGTTTTTATACATTACCGGTCAGCGTGCGGATGGTTACCACACGCTGCAAACGCTGTTTCAGTTTCTTGATTA
  1093                                                  HpaI          ORF2 —→
  CGGCGACACCATCAGCATTGAGCTTCGTGACGATGGGATATTCGTCTGTTAACGCCCGTTGAAGGC  GTG GAA CAT
                                                                      Val Glu His
  1168                                                                  1       3
  GAA GAT AAC CTG ATC GTT CGC GCA GCG CGA TTG TTG ATG AAA ACT GCG GCA GAC AGC GGG
  Glu Asp Asn Leu Ile Val Arg Ala Ala Arg Leu Leu Met Lys Thr Ala Ala Asp Ser Gly
                                                                             23
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1228
CGT CTT CCG ACG GGA AGC GGT GCG AAT ATC AGC ATT GAC AAG CGT TTG CCG ATG GGC GGC
Arg Leu Pro Thr Gly Ser Gly Ala Asn Ile Ser Ile Asp Lys Arg Leu Pro Met Gly Gly
1288                                                                        43
GGT CTC GGC GGT GGT TCA TCC AAT GCC GCG ACG GTC CTG GTG GCA TTA AAT CAT CTC TGG
Gly Leu Gly Gly Gly Ser Ser Asn Ala Ala Thr Val Leu Val Ala Leu Asn His Leu Trp
1348                                                                        63
CAA TGC GGG CTA AGC ATG GAT GAG CTG GCG GAA ATG GGG CTG ACG CTG GGC GCA GAT GTT
Gln Cys Gly Leu Ser Met Asp Glu Leu Ala Glu Met Gly Leu Thr Leu Gly Ala Asp Val
1408                    SphI                                                83
CCT GTC TTT GTT CGG GGG CAT GCC GCG TTT GCC GAA GGC GTT GGT GAA ATA CTA ACG CCG
Pro Val Phe Val Arg Gly His Ala Ala Phe Ala Glu Gly Val Gly Glu Ile Leu Thr Pro
1468 BamHI                                                                  103
GTG GAT CCG CCA GAG AAG TGG TAT CTG GTG GCG CAC CCT GGT GTA AGT ATT CCG ACT CCG
Val Asp Pro Pro Glu Lys Trp Tyr Leu Val Ala His Pro Gly Val Ser Ile Pro Thr Pro
1528                                                                        123
GTG ATT TTT AAA GAT CCT GAA CTC CCG CGC AAT ACG CCA AAA AGG TCA ATA GAA ACG TTG
Val Ile Phe Lys Asp Pro Glu Leu Pro Arg Asn Thr Pro Lys Arg Ser Ile Glu Thr Leu
1588         EcoRI                                                          143
CTA AAA TGT GAA TTC ACC CAG GCG TAC GGG CGG GCG AAT ACC AAA GGC GCT CCA TTC AGG
Leu Lys Cys Glu Phe Thr Gln Ala Tyr Gly Arg Ala Asn Thr Lys Gly Ala Pro Phe Arg
1648                                                                        163
AGA ACG GCG GTA AAG TGC AGG AGT CTG GGC AAA CTG CTT CTT GAA TGC GCG GGT AAA TGT
Arg Thr Ala Val Lys Cys Arg Ser Leu Gly Lys Leu Leu Leu Glu Cys Ala Gly Lys Cys
1708                                                                        183
CTG TTG AGA GTC GAA GCG GTA TTG CAG CGC GAT GTC CAG AAT CGG ACG CGC AGT CAG GCG
Leu Leu Arg Val Glu Ala Val Leu Gln Arg Asp Val Gln Asn Arg Thr Arg Ser Gln Ala
1768                                                        BalI            203
TAG TGCGACCGCCGATTTCGACAAACGACGAGCACGAATATACGCGCCAATAGCATGGCCAGTGACATCTTTAAACAT
***
1846
TCTCTGTAAGTGCCACTTGGAATAACCTGCTTCGCCGCTACATTGTCGAGCGACAGGGGCTGATCCAGATGACCTTCCA
1925                        NaeI
GCCTGATTAAAAGGTCGCGAATAATGCCGGC-3'
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Fig. 3. Nucleotide sequences of hemA and hemM regions and deduced aa sequences. Sequences were analyzed with the GENETYX program (SDC Software Development Co., Ltd., Tokyo, Japan). The aa sequence of the encoded protein is shown above the corresponding nt sequence. Numbers above the line are nt and numbers below the line are aa. The nt number 1 represents the SalI site in hemA (Fig. 2B). The direction of translation is indicated by arrows. SD represents the ribosome binding site. Asterisks indicate a stop codon. The complete nucleotide sequence has been submitted to EMBL/GenBank/DDBJ under accession no. D10264.

DISCUSSION

A contradiction as to the role of hemA in the ALA synthesis in E. coli has been arisen. Initially, Leang et al. (9) suggested that hemA was encoding ALA synthase. Recently, the hemA gene was isolated and its nt determined (3, 10, 17). The deduced aa sequence for the HemA protein shows no similarity to those of known ALA synthases. Avisar and Beale (1) demonstrated that E. coli forms ALA from glutamate via the C5 pathway, and that the strain SASX41B with the hemA mutation was characterized as the mutant deficient in glutamyl tRNA dehydrogenase. Thus, hemA in E. coli was re-designated as the gene encoding glutamyl tRNA dehydrogenase. However, the gene product has not yet been well characterized. Previous observations are in contrast to our finding that hemA conferred the limited growth to both hemL and hemM mutants in the C5 pathway. This finding suggests that hemA may not be located on the C5 pathway and may play a role in an alternative, minor, pathway for the biosynthesis of ALA. The weak growth of transformants suggest that the ALA formation via the alternative pathway is insufficient to recover the normal growth. This prediction may be supported by the studies on ALA in S. typhimurium. Elliott and Roth (5) proposed that hemA and hemL are required for one route of ALA synthesis and that a second, minor route for synthesis of ALA may operate in S. typhimurium.

The hemM appears to be a new gene that involved in the C5 pathway. It is possible that hemM could be another candidate for the gene encoding glutamyl tRNA dehydrogenase or its subunits. Further investigations will be required to elucidate the function

of hemM in the synthesis of ALA.

CONCLUSIONS

(1) Several E. coli Ala$^-$ mutants with defects in the C5 pathway were isolated. Genes responsible for the Ala$^-$ auxotrophy were cloned. By using these genes, the mutants isolated were classified into two groups, AlaA$^-$ and AlaB$^-$.

(2) The AlaA$^-$ mutants were determined to be defective in hemL that is located at 4 min on the chromosome and known to encodes GSA aminotransferase.

(3) The analysis of AlaB$^-$ mutants resulted in the finding of a new gene that was essential for the synthesis of ALA via the C5 pathway. The new gene that was designated hemM encoded an 207 aa ORF and was adjacent to hemA that is located at 27 min and thought to encode glutamyl tRNA dehydrogenase. The hemM and hemA were located 200 bp apart and transcribed divergently.

(4) The hemA gene has the ability to confer the weak growth to two different loci of mutations, hemL and hemM. These observations raise questions about the role of hemA in the synthesis of ALA. The product of hemA appears to act in an alternative, minor pathway that is distinct from the C5 pathway. The hemM gene could be another candidate for the gene encoding the glutamyl tRNA dehydrogenase, or its subunits.

SUMMARY

Several mutants of Escherichia coli that had lost their ability to synthesize δ-aminolevulinic acid (ALA) via the C5 pathway were isolated. Their defective loci were classified into two groups, AlaA$^-$ and AlaB$^-$. The genes that complemented these mutations were cloned. Nucleotide (nt) sequencing indicated that the gene that complemented AlaA$^-$ was identical to hemL that is located at 4 min on the E. coli chromosome and encodes glutamate 1-semialdehyde (GSA) aminotransferase. The gene complementing AlaB$^-$ contained an open reading frame (ORF) encoding a polypeptide of 207 aa, that was found to be a new gene, involved in the synthesis of ALA via the C5 pathway. Thus, we designated the gene hemM. The hemM gene was adjacent to hemA that is located at 27 min and previously thought to encode glutamyl tRNA dehydrogenase. However, we found that hemA complemented both the AlaA$^-$ (hemL) and AlaB$^-$ (hemM) mutants defective in the C5 pathway, although the transformants showed small colonies on the selective medium without ALA. These results suggest that hemA is not involved in the C5 pathway but controls a second, minor pathway for synthesis of ALA.

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第２章

Cloning and characterization of the glutamate 1-semialdehyde aminomutase gene from Xanthomonas campestris pv. phaseoli.

INTRODUCTION

δ-Aminolevulinic acid (ALA) is the first intermediate in the synthesis of hemes, chlorophylls, and billins. ALA is synthesized by either of two major biosynthetic pathways, namely the C5 and C4 pathways (15). In animal cells, yeast, fungi, and certain bacteria including non-sulfur purple bacteria, Rhodobacter, or Rhizobium, the synthesis of ALA is catalyzed by ALA synthase (EC 2.3.1.37) (7). The C5 pathway was found in plants (2), and some bacteria including Escherichia coli, Salmonella typhimurium, cyanobacteria, and anaerobic archaebacteria (1). In this pathway, ALA is synthesized in three steps, ligation of tRNA to glutamate, reduction of glutamyl-tRNA to form Glutamate 1-semialdehyde (GSA), and transamination of GSA to form ALA.

ALA-requiring mutants of E. coli and S. typhimurium have been identified on the basis of separate locations of two genes hemA and hemL that encode a structural component of glutamyl tRNA reductase (EC 6.1.1.17) (4, 11, 17) and GSA aminotransferase (EC 5.4.3.8) (9), respectively.

Recently, we isolated two classes of ALA defective mutants in E. coli (10). One class of mutants is identical to hemL, and another class of mutants is deficient in a new gene, hemM, that is involved in the C5 pathway of ALA synthesis. Characterizations of these mutants and their complemented genes raised questions about the role of hemA in the synthesis of ALA that hemA is not involved in the C5 pathway but controls a second, minor pathway for synthesis of ALA.

Xanthomonas species are gram-negative bacteria of agricultural and industrial importance due to their plant pathogenecity and their production of the useful polysaccharide, xanthan gum (18). The mechanisms by which bacteria cause plant disease and the regulation of biosynthetic pathway of xanthan gum are poorly understood at the genetic level. We have developed an efficient transformation and conjugation systems in Xanthomonas species (14). To investigate the ALA biosynthesis of phytopathogenic bacteria, we isolated the gene from X. campestris pv. phaseoli that complemented an ALA defective mutation in E. coli. The cloning and nucleotide sequence show that phytopathogenic bacterium, X. campestris pv. phaseoli, has a gene for GSA aminotransferase. Moreover the deduced amino acid sequence is highly homologous to those of HemL proteins from other organisms. It appears, therfore, that Xanthomonas synthesizes ALA via the C5 pathway.

MATERIALS AND METHODS

The bacterial strains and growth conditions. The strain of X. campestris pv. phaseoli used in this work was isolated in Thailand as a phytopathogenic bacterium for Phaseolus and stocked as the Hiroshima University Type Culture strain no. HUT8925. The bacterium was cultured in 2xYT [1.6% Bactotryptone, 1.0% Bacto yeast extract (Difco), 0.5% NaCl] at 28°C. E. coli strains I14 and I45 were AlaB$^-$ (hemM14) and AlaA$^-$ (hemL45), respectively. (10). The AlaA$^-$ mutant strain I45 has been proved to deficient in hemL that is located at 27 min on the E. coli chromosome and complemented with the hemL gene. E. coli JM109 was used for propagation of M13mp18 and M13mp19 phage vectors. E. coli cells were grown in LB [1% Bactotryptone, 0.5% Bacto yeast extract(Difco), 0.5% NaCl] or 2xYT at 37°C. The culture of Ala$^-$ strains of E. coli were supplemented with ALA at 50 μg/ml.

Enzymes and chemicals. Restriction endonuclease, T4 DNA Ligase, alkaline phosphatase, and Bal31 nuclease were purchased either from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Toyobo Co. Ltd. (Osaka, Japan). The other compounds used were standard commercial preparations.

Cloning of GSA aminotransferase gene. Cloning experiments were performed by the methods given in the manual of Maniatis et al. (12) using plasmid vector pUC18 (19). Chromosomal DNA from X. campestris pv. phaseoli, prepared by the method of Marmur (13), was partially digested with Sau3AI. DNA fragments from 4 to 10 kb

in length were isolated from an agarose gel, ligated to a BamHI-digested pUC18 with T4 DNA Ligase, after treatment with alkaline phosphatase, and used to transform into E. coli strain I45 ($HemL^-$). Transformants were selected on LB agar plates contained ampicillin (Ap) (100 μg/ml) but no ALA.

DNA sequencing. Sequencing reaction were performed by the M13 dideoxy-chain termination method (16) with Autoread T7 Sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). An automated laser fluorescence sequencing apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden) was used to determine the DNA sequences. The GenBank-EMBL-DDBJ accession number for the DNA sequence in Fig 2 is D12642.

Computer analysis. The nucleotide and amino acid sequences were analyzed with GENETYX programs (SDC Software Development Co. Ltd., Tokyo, Japan).

RESULTS AND DISCUSSION

Cloning of the gene from X. campestris pv. phaseoli that complements the hemL mutation of E. coli

Chromosomal DNA from X. campestris pv. phaseoli was partially digested with Sau3AI. The fragments were ligated with BamHI-digested pUC18, and the ligation mixture was used to transform the E. coli AlaA$^-$ (hemL) mutant I45. After a three-days incubation, four Ala$^+$ clones grew up on the selection plate prepared with Ap without ALA. The plasmids prepared from these transformants were confirmed to have the ability to retransform strain I45 to Ala$^+$, and the plasmids were designated pXP001 pXP002, pXP003 and pXP004. Two of the 4 clones, E. coli I45 carrying pXP003 and pXP004, grew in one day by contrast to other clones that required 2 to 3 days to form colonies on the selective plate.

Deletion analysis of the cloned genes

Restriction maps of the isolated DNA fragments were constructed (Fig. 1A). pXP001, pXP002, pXP003, and pXP004 contained an identical 1.5-kb SalI-SalI fragment, but the chromosomal DNA fragments in the plasmids pXP001 and pXP002 were inserted at the opposite direction to those in plasmid pXP003 and pXP004 (Fig. 1A). The differences in the strengths of complementation between pXP003 or pXP004 and pXP001 or pXP002 for Ala$^-$ seem to be due to the directions of the inserts of chromosomal fragments towards the lacZ gene on pUC18. Since pXP003 had the shortest insert of the 3.5-kb Sau3AI-Sau3AI fragment than others, this plasmid was

characterized in detail. To define the location of the gene on the 4-kb fragment that complemented the hemL mutation, the fragment was digested with restriction endonucleases and/or Bal31 nuclease. The fragments were subcloned into an appropriate site of the pUC18 vector. In the case of the fragments truncated with Bal31, the fragments were ligated at the blunt end to the HincII site of pUC18. The resultant plasmids were tested for their ability to complement the hem mutations. Figure 1B shows that the gene complementing hemL is on the pXC320 plasmid, which has an insert of less than 1.7 kb. None of the plasmids complemented the mutant strain I14 (hemM14) (data not shown).

Nucleotide sequence of the gene that complemented hemL

The 1.7-kb HincII/Bal31-KpnI fragment on pXC320 was digested with various restriction endonucleases and the resultant fragments were subcloned into phages M13mp18 and M13mp19. Seventeen overlapping fragments, which covered both strands of the entire fragment that complemented the hemL mutation, were sequenced. The complete nucleotide sequence of the sense strand and the deduced amino acid sequence are shown in Fig. 2.

One open reading frame (ORF), located between nucleotide 289 to 1575, was found. The ORF consists of 1287 bp with a putative ATG initiation codon that is preceded by a nucleotide with similarity to the -35 (TTccaA) and -10 (gATcAT) consensus sequences of E. coli and a potential ribosome-binding site (AGGA). The gene product was deduced to be a polypeptide of 45-kDa consisting of 429 amino acid residues.

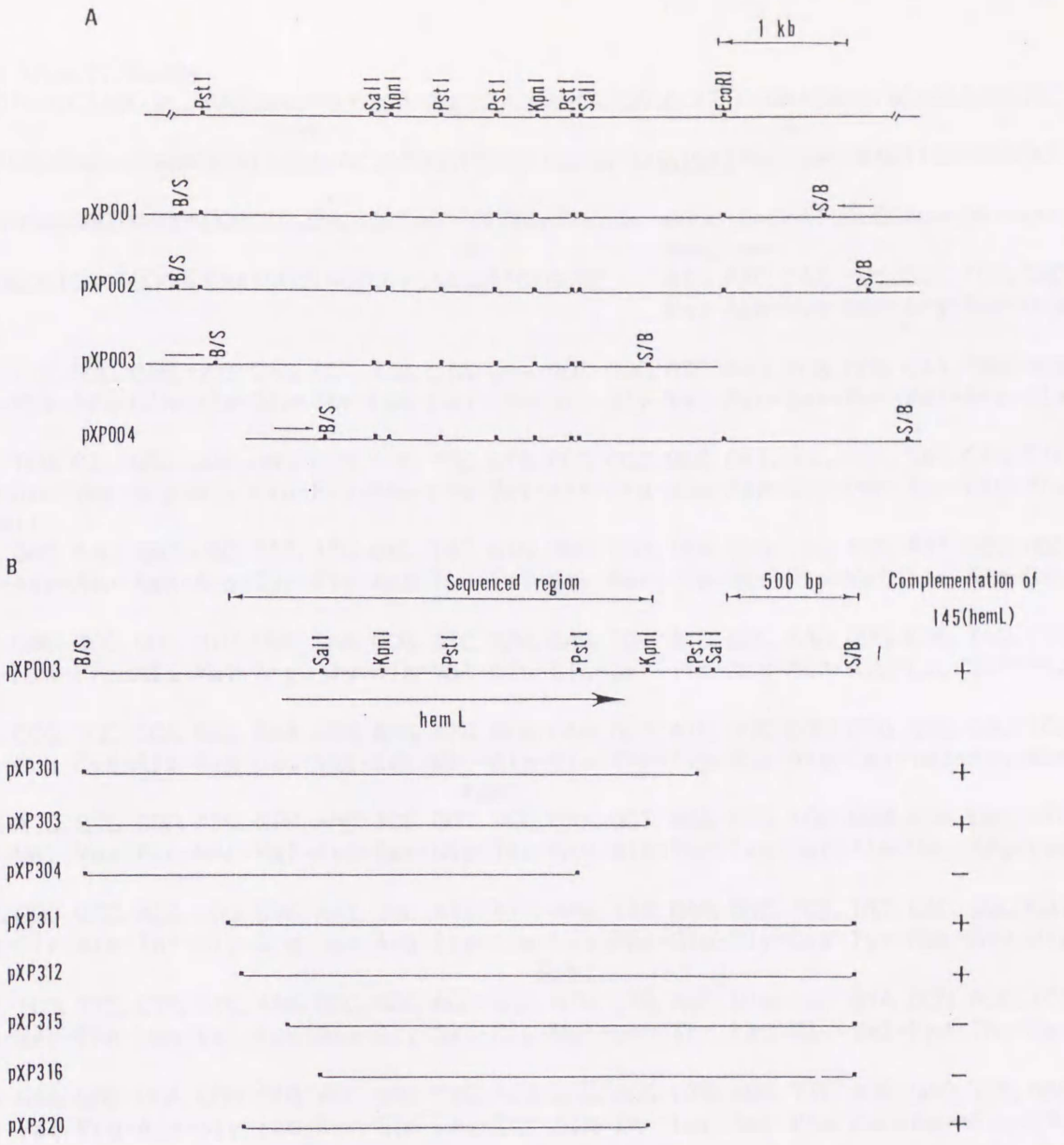

Fig 1 A restriction nuclease map of the cloned hemL region from X. campestris pv. phaseoli (A) and the deletion map of hemL region (B) and complementation of hemL mutant I45. The heavy line represents the X. campestris pv. phaseoli chromosome. The lines of plasmids indicate remaining DNA fragments. The directions of transcription of lacZ (A) and hemL (B) are indicated by arrows. Symbol + represents the complementation. S and B indicate Sau3AI and BamHI, respectively.

```
      *1 HincII/Bal31
      AGTCGGCAAGCGACAAACGAACGGCGCCAACGGCCACCGAAATCACACTTTCTGAACATCTACAGCCGGATGC
*74                          -35                                  -10
CAATGACTGGGCGCCATCAGCTCATTCCAAACTGTCGATCCCGTCGCGTTCGATCATAGGCAGCCAAGTTCCCCCACCC
*153
GCCCACTTCGACGGGTAGGCGGGCGTCCGACGGGTAGGCGCGCGGCCGCAGGCCTGACAATGGCGGACGCGATGCCC
*241                                        SD              hemL ──►
GCTGCCGGGCGTCGATGCCCCCAATCGGTACTCCTCAAGGATCTGCGC     ATG. AAC. CAT. TCC. CGC. TCC. CAC
                                                     Met-Asn-His-Ser-Arg-Ser-His
*310
GCC. CTG. TTC. GCC. CAG. GCG. CAG. ACT. TTG. CTA. CCG. GGC. GGC. GTC. AAC. TCG. CCG. GTA. CGG. GCG
Ala-Leu-Phe-Ala-Gln-Ala-Gln-Thr-Leu-Leu-Pro-Gly-Gly-Val-Asn-Ser-Pro-Val-Arg-Ala
*370
TTC. AAG. TCG. GTC. GGC. GGC. GAG. CCG. TTC. TTC. GTG. GCG. CGC. GCC. GAT. GGC. CCC. TAT. CTG. TTC
Phe-Lys-Ser-Val-Gly-Gly-Glu-Pro-Phe-Phe-Val-Ala-Arg-Ala-Asp-Gly-Pro-Tyr-Leu-Phe
*430  SalI
GAT. GTC. GAC. GAC. AAC. CGC. TAT. ATC. GAC. TAT. GTG. GGC. TCG. TGG. GGG. CCG. ATG. ATT. GCC. GGG
Asp-Val-Asp-Asp-Asn-Arg-Tyr-Ile-Asp-Tyr-Val-Gly-Ser-Trp-Gly-Pro-Met-Ile-Ala-Gly
*490
CAC. AAC. CAC. CCG. GCC. GTG. CGC. GAA. GCG. GTC. GAG. CAG. TCG. ATC. CGC. AAC. GGG. CTC. TCG. TTC
His-Asn-His-Pro-Ala-Val-Arg-Glu-Ala-Val-Glu-Gln-Ser-Ile-Arg-Asn-Gly-Leu-Ser-Phe
*550
GGT. GCA. CCG. TGC. GCA. GCC. GAA. GTG. ACG. ATG. GCG. CAG. ACC. ATC. GCC. CGG. CTG. GTG. CCC. TCC
Gly-Ala-Pro-Cys-Ala-Ala-Glu-Val-Thr-Met-Ala-Gln-Thr-Ile-Ala-Arg-Leu-Val-Pro-Ser
*610                                  KpnI
TGC. GAG. ATG. GTG. CGC. ATG. GTC. AAC. TCC. GGT. ACC. GAA. GCT. ACG. CTG. TCG. GCG. GTA. CGC. CTG
Cys-Glu-Met-Val-Arg-Met-Val-Asn-Ser-Gly-Thr-Glu-Ala-Thr-Leu-Ser-Ala-Val-Arg-Leu
*670
GCG. CGT. GGC. GCC. ACC. GGG. CGC. AAT. CGC. ATC. ATC. AAG. TTC. GAA. GGC. TGT. TAT. CAC. GGC. CAT
Ala-Arg-Gly-Ala-Thr-Gly-Arg-Asn-Arg-Ile-Ile-Lys-Phe-Glu-Gly-Cys-Tyr-His-Gly-His
*730                                       SphI
GGC. GAT. TCG. TTC. CTG. GTC. AAG. GCC. GGC. AGC. GGC. ATG. CTG. ACC. CTG. GGC. GTA. CCG. ACC. TCG
Gly-Asp-Ser-Phe-Leu-Val-Lys-Ala-Gly-Ser-Gly-Met-Leu-Thr-Leu-Gly-Val-Pro-Thr-Ser
*790
CCC. GGC. GTA. CCC. GCA. GGG. CTG. AGC. GAG. CTG. ACC. GCC. ACG. CTG. AGC. TTC. AAC. GAC. TTC. GAG
Pro-Gly-Val-Pro-Ala-Gly-Leu-Ser-Glu-Leu-Thr-Ala-Thr-Leu-Ser-Phe-Asn-Asp-Phe-Glu
*850
GGC. GCC. ACC. GCG. CTG. TTC. GAC. GAG. ATC. GGT. GCG. GAG. GTG. GCG. GCG. GTG. ATC. ATC. GAG. CCG
Gly-Ala-Thr-Ala-Leu-Phe-Asp-Glu-Ile-Gly-Ala-Glu-Val-Ala-Ala-Val-Ile-Ile-Glu-Pro
*910                                                    PstI
GTG. GTC. GGC. AAT. GCC. AAC. TGC. ATT. CCG. CCG. CAG. GCC. GGC. TAT. CTG. CAG. CAT. CTG. CGC. ACG
Val-Val-Gly-Asn-Ala-Asn-Cys-Ile-Pro-Pro-Gln-Ala-Gly-Tyr-Leu-Gln-His-Leu-Arg-Thr
*970
CTG. TGC. ACT. AGG. CAC. GGC. GCG. TTG. CTG. ATC. TTC. GAC. GAA. GTG. ATG. ACC. GGC. TTC. CGC. GTC
Leu-Cys-Thr-Arg-His-Gly-Ala-Leu-Leu-Ile-Phe-Asp-Glu-Val-Met-Thr-Gly-Phe-Arg-Val
*1030
GCG. CTC. GGT. GGT. GCG. CAG. GCG. CAT. TAC. GGC. GTG. ACC. CCG. GAC. CTG. ACC. ACC. TTC. GGC. AAG
Ala-Leu-Gly-Gly-Ala-Gln-Ala-His-Tyr-Gly-Val-Thr-Pro-Asp-Leu-Thr-Thr-Phe-Gly-Lys
*1090                SphI
ATC. ATC. GGC. GGC. GGC. ATG. CCG. GTG. GGC. GCC. TAC. GGC. GGC. CGC. CGC. GAC. TTG. ATG. GAG. CAG
Ile-Ile-Gly-Gly-Gly-Met-Pro-Val-Gly-Ala-Tyr-Gly-Gly-Arg-Arg-Asp-Leu-Met-Glu-Gln
*1150
GTT. GCT. CCG. GCC. GGG. CCG. ATC. TAC. CAG. GCC. GGC. ACC. TTG. TCG. GGC. AAC. CCG. GTG. GCC. ATG
Val-Ala-Pro-Ala-Gly-Pro-Ile-Tyr-Gln-Ala-Gly-Thr-Leu-Ser-Gly-Asn-Pro-Val-Ala-Met
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*1210
GCC. GCA. GGC. CTG. GCG. ATG. CTG. GAG. CTG. GTG. CAG. GAG. CCG. GGC. TTC. CAC. ACG. CGC. TTG. AGC
Ala-Ala-Gly-Leu-Ala-Met-Leu-Glu-Leu-Val-Gln-Glu-Pro-Gly-Phe-His-Thr-Arg-Leu-Ser
*1270          SphI
GAG. GCC. ACC. AGC. ATG. CTG. TGC. GAA. GGC. CTT. GAA. GAT. GCC. GCG. CGC. GCA. GCC. GGC. ATT. GCC
Glu-Ala-Thr-Ser-Met-Leu-Cys-Glu-Gly-Leu-Glu-Asp-Ala-Ala-Arg-Ala-Ala-Gly-Ile-Ala
*1330
GTG. ACC. ACC. AAC. CAG. GTT. GGC. GGC. ATG. TTC. GGG. CTG. TTC. TTC. ACC. GAC. GAC. GTC. GTG. GAG
Val-Thr-Thr-Asn-Gln-Val-Gly-Gly-Met-Phe-Gly-Leu-Phe-Phe-Thr-Asp-Asp-Val-Val-Glu
*1390
AGC. TAC. GCA. CAG. GCC. ACG. GCG. TGC. GAC. ATC. ACC. AGC. TTC. AAC. CGA. TTC. TTC. CAC. GCG. ATG
Ser-Tyr-Ala-Gln-Ala-Thr-Ala-Cys-Asp-Ile-Thr-Ser-Phe-Asn-Arg-Phe-Phe-His-Ala-Met
*1450 PstI
CTG. CAG. CGG. GGC. GTC. TAC. CTG. GCG. CCC. TCG. GCG. TAC. GAA. GCC. GGC. TTC. ATG. TCC. AGT. GCG
Leu-Gln-Arg-Gly-Val-Tyr-Leu-Ala-Pro-Ser-Ala-Tyr-Glu-Ala-Gly-Phe-Met-Ser-Ser-Ala
*1510
CAC. GAC. GAG. GCG. GTG. ATC. GAA. GCC. ACG. CTG. GCG. GCA. GCG. CGC. GAG. GCG. TTT. GCG. GAC. GTG
His-Asp-Glu-Ala-Val-Ile-Glu-Ala-Thr-Leu-Ala-Ala-Ala-Arg-Glu-Ala-Phe-Ala-Asp-Val
*1570
GCG. CGC. TGA  TCGGCTGGGAATCGGGAATGGGGAGTTGGGAATCGGTGCGCAATTAGCGCGGGGCGTTGAGGGTTT
Ala-Arg-***
*1630                                                                    KpnI
GCGGTGCGCGTGGCCGCGTGCCCGCAAGGCTGTGTGGCGGTGCCTCCGAACGCGCGCCACGGTACC
```

Fig 2 Nucleotide sequence and the deduced amino acid sequence of the hemL gene of X. campestris pv. phaseoli. The putative sequence of promoter and SD (ribosome-binding site) is underlined. The direction of translation is indicated by arrow. The complete nucleotide sequence has been submitted to EMBL/GenBank/DDBJ and accession no. is 12642.

Comparison of the HemL protein to those of other organisms.

The nucleotide sequences of the hemL gene encoding GSA aminotransferase from other organisms, including Barley (8), Salmonella typhimurium (5), E. coli and Synechococcus (9), have been reported.

Therefore, we compared the predicted amino acid sequence of the ORF of X. campestris pv. phaseoli with HemLs of other organisms, namely, barley, Synechococcus, S. typhimurium, and E. coli. The amino acid sequence of the ORF from X. campestris pv. phaseoli is homologous over its entire length to the other proteins with a total identity greater than 55% in all cases, Barley, 55.2%: Synechococcus, 57.7%; S. typhimurium, 62.4%; and E. coli, 62.5%. From the complementation of the hemL mutation and the similarity of amino acid sequence, we conclude that the cloned gene from X. campestris pv. phaseoli is hemL that encodes GSA aminotransferase. The hydrophobicity plot of the HemL protein from X. campestris pv. phaseoli also showed remarkably similar to the profiles of other HemLs (data not shown).

Aminotransferases require pyridoxal 5'-phosphate as a cofactor, and the pyridoxal 5'-phosphate bind to a lysine residue (6). Grimm et al. (9) suggested that the active-site lysine is invariably preceded by a leucine and a glycine residue. We found that in GSA aminotransferase of X. campestris pv. phaseoli six amino acid residues around the putative active site lysine residue (Lys^{267}) were conserved and that the Phe^{265} was substituted for leucine. The analysis of second structure of the predicted HemL protein of X. campestris pv. phaseoli with Chou-Fasman

equation (3), showed that the predicted pyridoxal 5'-phosphate-binding region could form a part of β-strand, as predicted for HemL proteins from other sources. Thus, the substitution of Phe^{265} for leucine seems not to affect the active structure of the HemL protein.

Thus, a phytopathogenic bacterium, X. campestris pv. phaseoli, has GSA aminotransferase that is involved in the C5 pathway for synthesis of ALA.

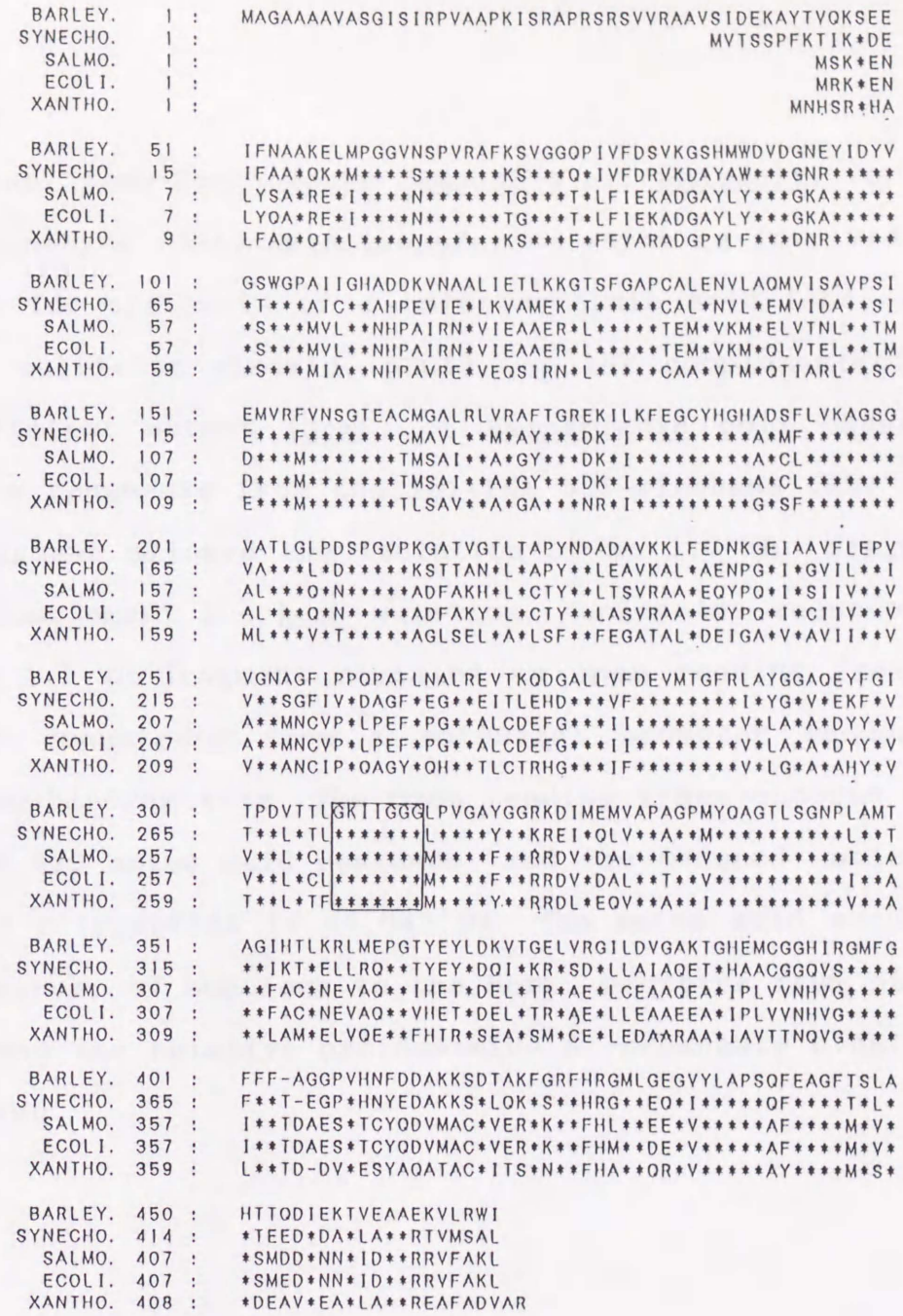

Fig 3 Comparison of the amino acid sequence of the HemL protein from X. campestris pv. phaseoli with those of other organisms. Amino acid residues conserved in all HemL proteins are indicated by asterisks. A putative pyridoxal 5'-phosphate-binding site in various HemL is boxed. BARLEY, barley; SYNECHO, Synechococcus; SALMO, S. typhimurium; ECOLI, E. coli; XANTH, X. campestris pv. phaseoli.

SUMMARY

The gene, from Xanthomonas campesris pv. phaseoli, for glutamate 1-semialdehyde (GSA) aminotransferase which is involved in the C5 pathway for synthesis of δ-aminolevulinic acid (ALA) was cloned onto a multicopy plasmid, pUC18, by the complementation for an ALA-deficient mutant (hemL) of Escherichia coli. Subcloning of deletion fragments from the initial 3.5-kirobase (kb) chromosomal fragment allowed the isolation of an 1.7-kb fragment which could complement the hemL mutation. Nucleoride sequence analysis of the 1.7-kb fragment revealed an open reading frame that is located downstream from a potential promoter sequence and a ribosome-binding site. The open reading frame encodes a polypeptide of 429 amino acid residues, and the deduced molecular mass of this polypeptide is 45,043 Da. The amino acid sequence shows high degree of homology to the HemL proteins from other organisms, and the putative pyridoxamine 5'-phosphate binding site is conserved.

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第 3 章

Cloning and characterization of the gene for glutamate 1-semialdehyde 2,1-aminomutase which is involved in the δ-aminolevulinic acid synthesis in Propionibacterium freudenreichii

INTRODUCTION

Propionibacterium species are Gram-positive bacteria that are used in several dairy and fermentation systems, including production of cobalamin (e.g., vitamin B_{12}) and propionic acid (20). Advances in fermentation technology and product separation, together with the development of genetically engineered strains, may result in improvements in performance that are necessary to make these fermentations more economically competitive and may also improve the quality of current products. The need for improved strains for industrial fermentations has prompted genetic studies of these bacteria.

δ-Aminolevulinic acid (ALA) is the first intermediate in the synthesis of hemes, cobalamin, and chlorophylls. In Propionibacterium, ALA is a precursor of heme and cobalamin syntheses (14). ALA is synthesized by either of two major biosynthetic pathways, namely, the C5 and C4 pathways (20). In animal cells, yeast, fungi, and in certain bacteria including non-sulfur purple bacteria, Rhodobacter, and Rhizobium, the synthesis of ALA is catalyzed by ALA synthase (EC 2.3.1.37) (8). The C5 pathway has been found in plants (2), and in some bacteria, including Escherichia coli (4, ,10, 12, 13, 16, 24), Salmonella typhimurium (6), cyanobacteria (10), anaerobic archaebacteria (1), and Bacillus subtilis (11). In this pathway, ALA is synthesized in three steps: ligation of tRNA to glutamate, reduction of glutamyl-tRNA to generate glutamate 1-semialdehyde (GSA), and transamination of GSA to generate ALA.

ALA-requiring mutants of E. coli and S. typhimurium have been

identified on the basis of separate locations of two genes, hemA and hemL, that encode the structural component of glutamyl tRNA reductase (EC 6.1.1.17) (4, 16, 24) and that of GSA aminomutase (EC 5.4.3.8) (5, 10, 12), respectively, although the role of hemA is still obscure (12).

To investigate the biosynthesis of ALA in a vitamin B_{12}-producing bacterium, we isolated the gene from P. freudenreichii that complemented a hemL mutation in E. coli. The complementation and the nucleotide sequence of the gene show that P. freudenreichii has a gene for GSA aminomutase. Moreover, the deduced amino acid sequence is highly homologous to those of HemL proteins from other organisms. It appears, therefore, that Propionibacterium synthesizes ALA via the C5 pathway.

MATERIALS AND METHODS

A vitamin B_{12}-producer, P. freudenreichii IFO 12424 (IFO, Institute for Fermentation, Osaka) was cultured in glucose complete medium [12.5 g Polypepton (Wako Pure Chemicals Co. Ltd. Osaka Japan), 11.0 g vitamin-free casamino acids, 2.5 g yeast extract, 2.25 g $NaH_2PO_4.2H_2O$, 1.76 g K_3PO_4, 0.5 g L-cysteine, 0.4 g $MgCl_2.6H_2O$, 0.01 g $FeSO_4.7H_2O$, 44 mg Ca-pantothenate, 0.3mg biotin, 10.0 g glucose, 5.0 g KNO_3, 0.018 g $CoCl_2.6H_2O$, 0.001 g Na_2MoO_4 per liter, pH 7.0) at 28°C for three days. E. coli strains SASX41B (hemA41, 23) and I45 (hemL45, 12) were defective in the C5 pathway of ALA synthesis. E. coli JM109 was used for propagation of M13mp18 and M13mp19 phage vectors. E. coli cells were grown in LB medium [1% Bactotryptone, 0.5% Bacto yeast extract (Difco, Detroit, MI.), 0.5% NaCl, pH 6.8] or 2 x YT [1.6% Bactotryptone, 1.0% Bacto yeast extract (Difco), 0.5% NaCl, pH 6.8] at 37°C. The cultures of Ala^- strains of E. coli strains were supplemented with ALA at 50 μg/ml.

Restriction endonucleases, T4 DNA ligase, alkaline phosphatase, and Bal31 nuclease were purchased either from Takara Shuzo Co. Ltd. (Kyoto, Japan) or Toyobo Co. Ltd. (Osaka, Japan). All other compounds used were standard commercial preparations. Cloning experiments were performed by the methods given in the manual of Maniatis et al. (17) using plasmid vector pUC18 (26). Chromosomal DNA from P. freudenreichii, prepared by the method of Marmur (18), was partially digested with Sau3AI. DNA fragments from 4 to 9 kb in length were isolated from an agarose gel, ligated to BamHI- digested pUC18 that was treated with alkaline phosphatase,

and used to transform E. coli strain I45 (hemL). Transformants were selected on LB agar plates that contained ampicillin (Ap) (100 μg/ml) but no ALA.

Sequencing reactions were performed by the M13 dideoxy-chain termination method (21) with an Autoread T7 Sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). An automated laser fluorescence sequencing apparatus (Pharmacia LKB Biotechnology) was used to determine the DNA sequences. The GenBank-EMBL-DDBJ accession number for the DNA sequence in Figure 2 is D12543. The nucleotide and amino acid sequences were analyzed with GENETYX programs (SDC Software Development Co. Ltd., Tokyo, Japan).

RESULTS

Chromosomal DNA from *P. freudenreichii* was partially digested with *Sau*3AI. The fragments were ligated with *Bam*HI-digested pUC18, and the ligation mixture was used to transform the *E. coli* HemL$^-$ mutant I45. After a two-day incubation, two Ala$^+$ clones grew up on the selection plate prepared with Ap but without ALA. The two plasmids prepared from these transformants were confirmed to have the ability to reconvert strain I45 to Ala$^+$, and the two plasmids were designated pFR001 and pFR002, respectively.

Restriction maps of the isolated DNA fragments were constructed (Fig. 1). pFR001 and pFR002 had an identical 3.3-kb *Sau*3AI fragment. Since pFR002 had the shorter chromosomal insert, this plasmid was characterized in detail. To define the location of the gene on the 3.3-kb fragment that complemented the *hemL* mutation, the fragment was digested with restriction endonucleases and/or Bal31 nuclease. The fragments were subcloned into an appropriate site of the pUC18 vector. In the case of the fragments truncated with Bal31, the fragments were ligated at the blunt end to the *Hin*cII site of pUC18. The resultant plasmids were tested for their ability to complement the *hem* mutations. Figure 1 shows that the gene that complemented *hemL* was on the pFR222 plasmid, which has an insert of less than 1.9 kb. None of the plasmids complemented the mutant strain SASX41B (*hemA*41) (data not shown).

The 1.9-kb *Bcl*I/*Bam*HI fragment on pFR222 was digested with various restriction endonucleases and the resultant fragments were subcloned into phages M13mp18 and M13mp19. Twenty overlap-

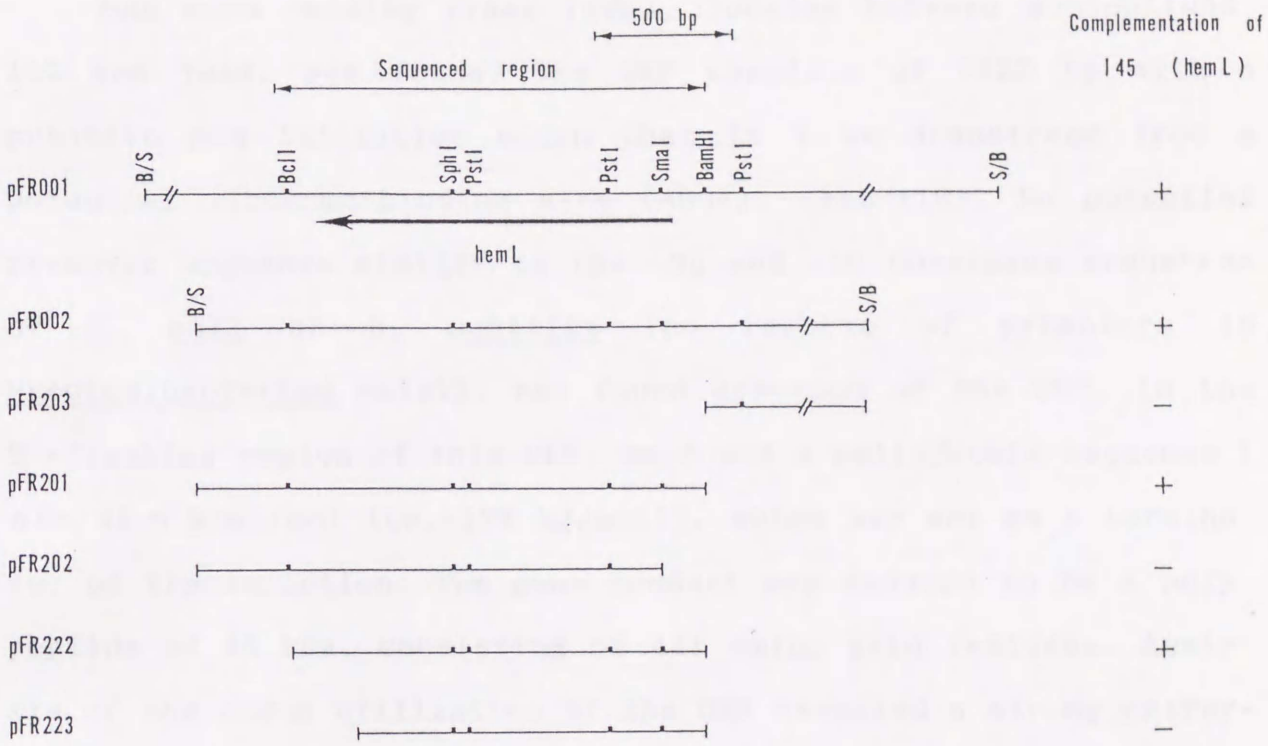

Fig. 1. A deletion map of the cloned hemL region from P. freudenreichii and the complementation of the hemL mutant E. coli I45. The heavy line represents the chromosome of P. freudenreichii. The lines representing the plasmids indicate the remaining fragments of DNA. The direction of transcription of hemL is indicated by an arrow. The symbol + represents complementation. S and B indicate Sau3AI and BamHI, respectively.

ping fragments, which covered both strands of the entire fragment that complemented the hemL mutation, were sequenced. The complete nucleotide sequence of the sense strand and the deduced amino acid sequence are shown in Figure 2.

One open reading frame (ORF), located between nucleotides 122 and 1444, was found. The ORF consists of 1323 bp with a putative ATG initiation codon that is 7 bp downstream from a potential ribosome-binding site (AGGA), (113-116). No potential promoter sequence similar to the -35 and -10 consensus sequences of E. coli or B. subtilis (no reports of promoters in Propionibacterium exist), was found upstream of the ORF. In the 3'-flanking region of this ORF, we found a palindromic sequence [ΔG=-42.6 kcal/mol (ca.-178 kJ/mol)], which may act as a terminator of transcription. The gene product was deduced to be a polypeptide of 46 kDa, consisting of 441 amino acid residues. Analysis of the codon utilization of the ORF revealed a strong preference for guanine and cytosine at the third position of the codon (90%), consistent with the high G+C content of the genome of Propionibacterium (25). The overall G+C content of the ORF is 70%.

Since the direction of transcription of the ORF is opposite to that of the lac promoter on pFR201, we constructed a plasmid in which the ORF was located downstream from the lac promoter. The resultant plasmid, pFR204, complemented the E. coli I45 mutant (hemL), and the formation of colonies was observed within one day, whereas E. coli cells transformed by pFR201 formed colonies that were visible after only 2 to 3 days. These results suggest that in the region upstream of the ORF, there may exist

```
                                    BamHI 1
                                    GGATCCGGCCCGCGCCTGGGCGACCGGTCGTCGGGCCGGCCC

43                                                                      SD
CGCTGCGGGCAGACCCTGAGAATGCGAGGGGCAAGGCCATGCCCGCTGCCGGAATGCCCGGCCCGACAAGGAAGAACCC

122 hemL──►                                                    SmaI
ATG AGT GTC AGT GAC GAA TTG TTC GCA GAA GCC CTC AAG GTC ATG CCC GGG GGC GTC TCC
Met Ser Val Ser Asp Glu Leu Phe Ala Glu Ala Leu Lys Val Met Pro Gly Gly Val Ser

182
TCG CCG GTG CGC GCC TAC CGC AGT GTC GGG GGC ACG CCA CGC TTC GTG AAG CGG GCG CTG
Ser Pro Val Arg Ala Tyr Arg Ser Val Gly Gly Thr Pro Arg Phe Val Lys Arg Ala Leu

242                                                            PvuII
GGT TCC CAC ATC GTC GAC GTC GAC GAC AAG CGC TAT GTC GAC CTG GTG TGC AGC TGG GGG
Gly Ser·His Ile Val Asp Val Asp Asp Lys Arg Tyr Val Asp Leu Val Cys Ser Trp Gly

302                                                        PstI
CCG ATG ATC GCC GGC CAC GCC CAC CCC GAG GTG GTG GCC GCC GTG CTG CAG GCC GTT GCC
Pro Met Ile Ala Gly His Ala His Pro Glu Val Val Ala Ala Val Leu Gln Ala Val Ala

362
GAT TCC ACC TCG TTC GGC GCG CCC AGT GAG GTG GAG CTG CGG CTC GCC CAG GCC GTC GTC
Asp Ser Thr Ser Phe Gly Ala Pro Ser Glu Val Glu Leu Arg Leu Ala Gln Ala Val Val

422
GCC CGC ATG GGC GGC GCC ATC GAC AAG GTG CGC TTC ACC TGT TCG GGC ACC GAG GCC GTG
Ala Arg Met Gly Gly Ala Ile Asp Lys Val Arg Phe Thr Cys Ser Gly Thr Glu Ala Val

482
ATG ACC GCG GCC CGA CTG GCG CGC GGC ATC ACG AAG CGA CCG CTG CTG GTC AAG TTC GTC
Met Thr Ala Ala Arg Leu Ala Arg Gly Ile Thr Lys Arg Pro Leu Leu Val Lys Phe Val

542
GGC TGC TAC CAC GGC CAT TCC GAT TCC TTC CTG GTG TCG GCC GGC TCC GGG GTG GCA AGC
Gly Cys Tyr His Gly His Ser Asp Ser Phe Leu Val Ser Ala Gly Ser Gly Val Ala Ser

602
CTG GGC CTG CCC GAT TCG CCC GGC GTG CCC AAG GAG GTC GCC GGC GAC ACG GTC GCC CTG
Leu Gly Leu Pro Asp Ser Pro Gly Val Pro Lys Glu Val Ala Gly Asp Thr Val Ala Leu

662
CCC TAT GGG CGC ATC GAC ATG GTC GAG GAG CTG TTC GCC GAG CGT GGC GAC CAG GTG GCG
Pro Tyr Gly Arg Ile Asp Met Val Glu Glu Leu Phe Ala Glu Arg Gly Asp Gln Val Ala

722
GCC ATC GTC ACC GAG GGC GTC CCG GCG AAC ATG GGC GTC ATC GTG CCG CCC GAG GGC TTC
Ala Ile Val Thr Glu Gly Val Pro Ala Asn Met Gly Val Ile Val Pro Pro Glu Gly Phe

782
AAC CGT CGC CTG CAT GAC ATC GCC CAC GCC CAC GGC GCC CTT CTC ATC CAG GAC GAG GTG
Asn Arg Arg Leu His Asp Ile Ala His Ala His Gly Ala Leu Leu Ile Gln Asp Glu Val
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842                                                    PstI
CTC ACC GGT TTC CGG CTG AGC CCC ACC GGC GCC TGG GGA CTG CAG GGG GCG AAG GAG GGG
Leu Thr Gly Phe Arg Leu Ser Pro Thr Gly Ala Trp Gly Leu Gln Gly Ala Lys Glu Gly

902                                                          SphI
TGG ACT CCC GAC CTG TTC ACC TTC GGC AAG GTG ATC GGT GGC GGC ATG CCA CTG GCG GCC
Trp Thr Pro Asp Leu Phe Thr Phe Gly Lys Val Ile Gly Gly Gly Met Pro Leu Ala Ala

962                  PvuII
GTG GGT GGT TCG GCG CAG CTG ATG GAC TAC CTG GCC CCC GAG GGG CCC GTC TAC CAG GCG
Val Gly Gly Ser Ala Gln Leu Met Asp Tyr Leu Ala Pro Glu Gly Pro Val Tyr Gln Ala

1022                                                   BalI
GGC ACC CTG TCG GGT AAC CCG GCT GCC TGC GCG GCG GGG CTG GCC ACG CTT GCC CTC ATG
Gly Thr Leu Ser Gly Asn Pro Ala Ala Cys Ala Ala Gly Leu Ala Thr Leu Ala Leu Met

1082
GAC GAC GCC GCC TAC TCC CGA CTG GAC GCC ACC GCC GAC CGG GTC TCG GCG ATG GCC GAT
Asp Asp Ala Ala Tyr Ser Arg Leu Asp Ala Thr Ala Asp Arg Val Ser Ala Met Ala Asp

1142
GCG GCG CTG GAG TCC GCC GGG GTG CCC CAC CGG ATC AAC AAG GTC TCC AAC CTG TTC AGC
Ala Ala Leu Glu Ser Ala Gly Val Pro His Arg Ile Asn Lys Val Ser Asn Leu Phe Ser

1202
GTC TTC CTC ACC GAC GCC CCG GTG ACT GAC TTC GCC TCG GCG TCC AAG CAG GAC ACC AAG
Val Phe Leu Thr Asp Ala Pro Val Thr Asp Phe Ala Ser Ala Ser Lys Gln Asp Thr Lys

1262
GCG TTC TCG CGC TTC TTC CAC GCG GCA CTC GAT GCC GGC CTG TGG CTG GCC CCC AGC GGC
Ala Phe Ser Arg Phe Phe His Ala Ala Leu Asp Ala Gly Leu Trp Leu Ala Pro Ser Gly

1322   StuI
TTC GAG GCC TGG TTC TGC TCC ACC GCC CTG GAT GAC GAC GAC CTT GAG GTC ATC GAC GCC
Phe Glu Ala Trp Phe Cys Ser Thr Ala Leu Asp Asp Asp Asp Leu Glu Val Ile Asp Ala

1382
GGC CTG CAC AAG GCT GCA CAG GCG GCC GCC CAG GGC CTT TCC TCG TTG GAG GAT GTG CGC
Gly Leu His Lys Ala Ala Gln Ala Ala Ala Gln Gly Leu Ser Ser Leu Glu Asp Val Arg

1442
CGC TGA TCGGTGTCTCGCCAGGCGGTGGCAATTGCGGAGTGGGCGCGTTTCCGGCTTGTTCGCCGGACGCGCCCACT
Arg ***

1519                          BclI
GCCATGTCCGAAACTGTTCAAATGGTGATCA
```

Fig. 2. Nucleotide sequence and the deduced amino acid sequence of the hemL gene of P. freudenreichii. SD, a ribosome-binding site and the putative terminator of transcription are underlined. The direction of translation is indicated by an arrow. The complete nucleotide sequence has been submitted to EMBL/GenBank/DDBJ and the accession no. is D12543.

an alternative promoter sequence that can be recognized, but only weakly, by the RNA polymerase of E. coli.

The nucleotide sequences of the hemL genes encoding GSA aminomutase from other organisms have been reported. Therefore, we compared the predicted amino acid sequence of the ORF of P. freudenreichii with those of HemL proteins of other organisms, namely, barley (9), Synechococcus (10), S. typhimurium (5), E. coli (10, 13), B. subtilis (11), and Xanthomonas campestris pv. phaseoli (19). The amino acid sequence of the ORF from P. freudenreichii is somewhat homologous over its entire length to the other proteins with a total identity greater than 45% in all cases, as follows: barley, 46.8%; Synechococcus, 48.8%; S. typhimurium, 46.2%; E. coli, 46.4%; B. subtilis, 46.5%; and X. campestris pv. phaseoli, 48.9%. From the complementation of the hemL mutation and the similarity to other amino acid sequences, we conclude that the cloned gene from P. freudenreichii is hemL and encodes GSA aminomutase. The hydrophobicity plot of the HemL protein from P. freudenreichii was also remarkably similar to the profiles of other HemL proteins (data not shown).

Aminomutases require pyridoxal 5'-phosphate (PLP) as a cofactor, and the PLP can bind to a lysine residue (7). The active-site lysine is invariably preceded by a leucine and a glycine residue. We found that in the GSA aminomutase of P. freudenreichii phenylalanine replaces leucine, as in the enzyme from X. campestris pv. phaseoli. The analysis of the secondary structure of the predicted HemL protein of P. freudenreichii, using the Chou-Fasman equation (3), showed that the predicted

PLP-binding region could form a part of β-strand, as predicted for HemL proteins from other sources. Thus, the substitution of phenylalanine for leucine seems not to affect the structure of the HemL protein.

DISCUSSION

The Gram-positive bacterium, *B. subtilis* has the *hemAXCDBL* gene cluster that includes *hemA*, which encodes glutamyl tRNA reductase, *hemX*, which encodes a hydrophobic protein of unknown function; *hemC*, which encodes hydroxylmethylbilane synthase; *hemD*, which encodes uroporphyrinogen III synthase; *hemB*, which encodes porphobilinogen synthase; and *hemL*, which encodes GSA aminomutase. These six genes may constitute a *hem* operon, encoding the enzymes required for the synthesis of uroporphyrinogen III from glutamate (11). In this report, we have shown that a second Gram-positive bacterium, *P. freudenreichii*, has a GSA aminomutase that is involved in the C5 pathway for the synthesis of ALA. It will be of interest to determine the existence of *hem* operon in *P. freudenreichii* since the bacterium is used to produce cobalamin via ALA as a precursor (14), and the only other genetic information available for this species is the nucleotide sequence of the gene for phosphofructokinase (15).

SUMMARY

The gene, from Propionibacterium freudenreichii, for glutamate 1-semialdehyde (GSA) 2, 1-aminomutase, which is involved in the C5 pathway for synthesis of δ-aminolevulinic acid (ALA), a precursor in heme and cobalamin biosynthesis, was cloned onto a multicopy plasmid, pUC18, via complementation of an ALA-deficient mutant (hemL) of Escherichia coli. Subcloning of fragments from the initial 3.3-kilobase (kb) chromosomal fragment allowed the isolation of a 1.9-kb fragment which could complement the hemL mutation. Nucleotide sequence analysis of the 1.9-kb DNA fragment revealed an open reading frame (ORF) that was located downstream from a potential ribosome-binding site. The ORF encoded a polypeptide of 441 amino acid residues, and the deduced molecular mass of this polypeptide is 45,932 Da. A high G + C content (70%) of the codons of the ORF was found and was consistent with the taxonomic features of species of Propionibacterium. The amino acid sequence showed a high degree of homology to the HemL proteins from other organisms, and a putative binding site for pyridoxal 5'-phosphate was conserved, with the exception of a single substitution of phenylalanine for leucine. These results suggest that ALA is synthesized via the C5 pathway in a producer of vitamin B_{12}, P. freudenreichii.

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第４章　総括

δ－アミノレブリン酸(ALA)は、ヘム、クロロフィル、チトクロム等テトラピロール化合物の最初の前駆体であり、生物に普遍的に存在する重要なアミノ酸である。さらに選択除草剤としての用途も見いだされ農薬としても注目を集めている。この生合成は、従来グリシンとスクシニルCoAからALAシンターゼによって生合成されるShemine(C4)経路によって生合成されると考えられてきた。近年、植物においてグルタミン酸から生合成されるC5経路が発見され、大腸菌のALA生合成もC5経路によるとされてきた。しかしながら、別経路の存在の可能性も示唆されるなどより詳細な解析が求められている。本研究においては、細菌(E. coli, Xanthomonas, Propionibacterium)のALA生合成とその遺伝子レベルでの発現調節機構を解明することを目的とした。以下、本論文を要約する。

第１章では大腸菌のALA生合成機構について述べた。まず、大腸菌のALA生合成がどちらの経路によって行われているかを調べた。C5経路の活性は検出できたがShemine(C4)経路の活性は検出できなかった。大腸菌においてはALA生合成はC5経路により合成されているものと考えられる。ALA生合成に関わる遺伝子を単離するため、次に変異処理によってALA要求性変異株６株を取得した。これらの変異はすべてグルタミン酸からのALA生合成(C5経路)を欠損していた。変異を相補する遺伝子をクローニングして各変異株に対する相補性を調べたところ、これらの変異は少なくとも２種類に大別された(AlaA$^-$, AlaB$^-$)。

AlaA$^-$株を相補する遺伝子(alaA)は大腸菌染色体上約４分の位置にあった。一方AlaB$^-$株を相補する遺伝子(alaB)は約２７分の位置にあった。遺伝子の欠失解析を行ったところ”不均一な”相補性を示した。さらにalaB遺伝子に隣接して存在するalaC遺伝子はAlaA$^-$株、AlaB$^-$株どちらも相補した。

取得した３つの遺伝子の全塩基配列を決定した。

alaA遺伝子は1278bpよりなり推定分子量４万５千の蛋白をコードしていた。この遺伝子はホモロジー検索の結果 hemL遺伝子であった。従ってAlaA$^-$株はhemLであると考えられる。

alaB遺伝子は621bpよりなり推定分子量２万３千の蛋白をコードしていた。この遺伝子知の遺伝子とのホモロジーはなく新規の遺伝子であった。この遺伝子hemMと名ずけた。

alaC遺伝子は1254bpよりなり推定分子量４万６千の蛋白をコードしていた。この遺伝子はホモロジー検索の結果意外にも hemA遺伝子と同一であった。hemA遺伝子はC5経路におけるglutamyl-tRNA reductase 欠損変異株を相補できる遺伝子として単離され、そのためglutamyl-tRNA reductase をコードするものと考えられてきた。しかしながら、本研究においてはhemAと呼ばれている遺伝子はhemL、hemMどちらの変異も相補出来ることから、glutamyl-tRNA reductase をコードする

だけでなくC5経路以外の別経路上の遺伝子としても働いているものではないかと推定された。

さらにこれら3つの遺伝子を変異株、親株に導入しALA生合成能を測定したところhemM、hemA単独で導入した変異株は親株と同程度まで回復したのに対しhemM-hemA領域を導入するとALA生合成能は数倍上昇した。hemM-hemA遺伝子は隣接して逆向きに存在しており両遺伝子間で何らかの調節機構が存在しているものと考えられた。

第2章では植物病原細菌であるXanthomonas campestris pv. phaseoliのALA生合成に関する遺伝子の単離と解析について述べた。大腸菌のhemL変異株を宿主とし相補できる遺伝子を単離した。本遺伝子の塩基配列を決定したところ、推定分子量45,043の蛋白をコードしていた。ホモロジー検索を行った結果、GSA aminomutaseと同定した。従って、Xanthomonas属においてC5経路の存在が確認できた。

第3章ではビタミンB12生産菌であるPropionibacteriumよりALA生合成に関わる遺伝子の単離と解析について述べた。大腸菌のhemL変異株を宿主とし相補できる遺伝子を単離した。本遺伝子の塩基配列を決定したところ、推定分子量45,932の蛋白をコードしていた。ホモロジー検索を行った結果、GSA aminomutaseと同定した。Propionibacterium属においてはC4経路とC5経路の共存が報告されているが、このうちC5経路の酵素の遺伝子が確認できた。

本研究において3つの細菌からC5経路上のGSA aminomutaseをcodeするhemL遺伝子を単離した。推定されるアミノ酸配列を次のFIG.5.1に示す。GSA aminomutaseはピリドキサールリン酸(PLP)を補酵素とする酵素でありその結合部位も共通して見られた。また、他のPLPを補酵素とする酵素ともその結合部位を比較した(FIG.5.2)。PLP結合部位は従来leucine、glycineに続くlysineに結合するとされていたが、Xanthomonas campestris pv. phaseoli、Propionibacterium freudenreichiiのHemLにおいてはleucineがphenylalanineに変化しており、必ずしもleucineがPLPの結合に必要でないことが明かとなった。

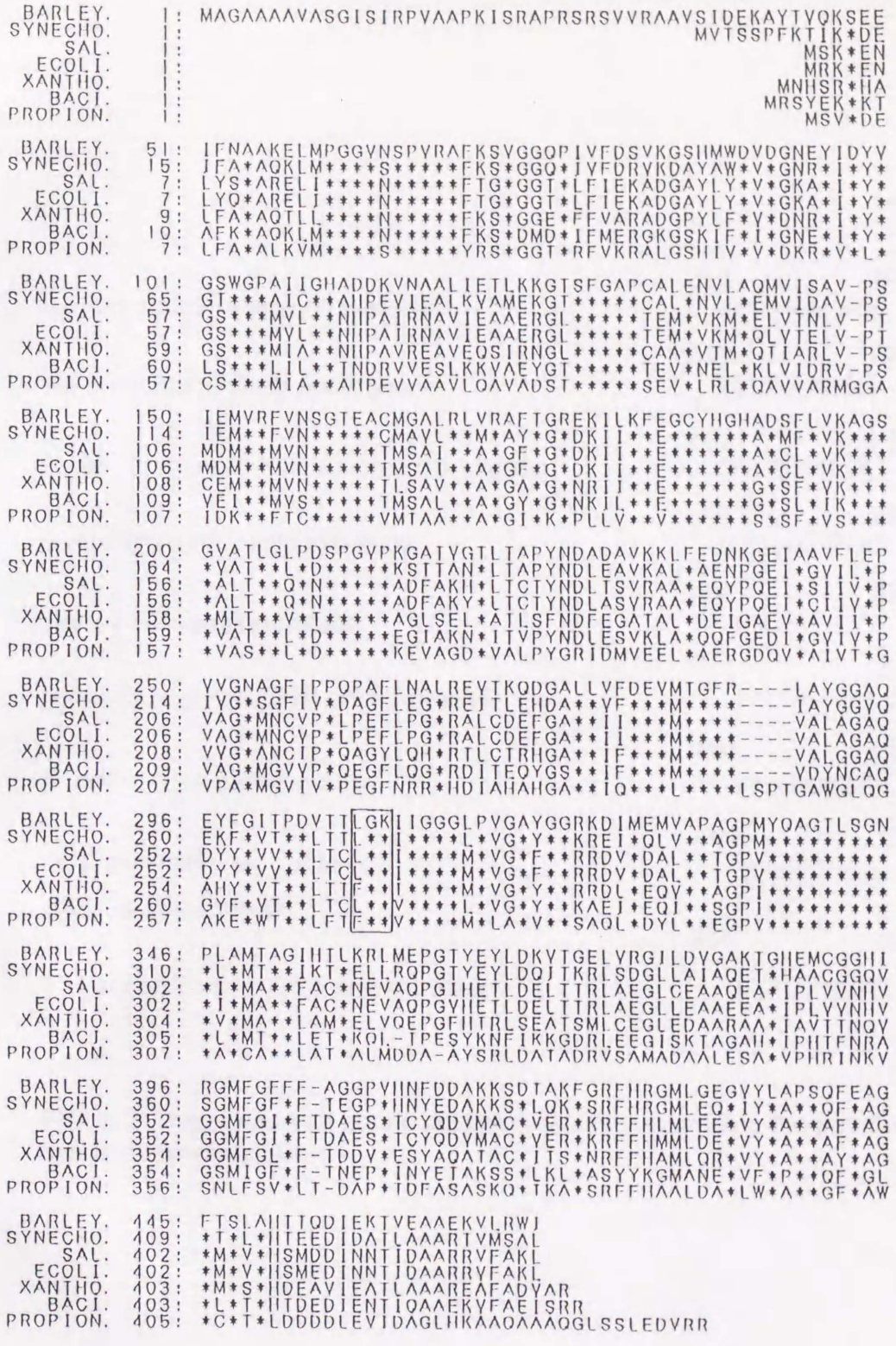

FIG. 5.1 Comparison of the deduced amino acid sequence of the HemL protein. Amino acid residues conserved in all HemL proteins are indicated as asterisks. A putative pyridoxal 5'-phoshate-binding site in various HemL proteins is boxed. BARLEY, barley; SYNECHO, *Synechococcus*; SAL, *S. typhimurium*; ECOLI, *E.coli*; XANTHO, *X. campestris* pv. *phaseoli*; BACI, *B. subtilis*; PROPION, *P. freudenreichii*

human ornithine aminotransferase	288 VLLGKALSG	296
rat ornithine aminotransferase	288 VLLGKALSG	296
E.coli 2-amino-3-ketobutyrate CoA ligase	240 GTLGKALGG	248
5-aminolevulinic acid synthase	386 GTLGKAFGC	394
barley GSA aminomutase	268 TTLGKIIGG	276
Synechococcus GSA aminomutase	271 TTLGKIIGG	279
E. coli GSA aminomutase	269 ICLGKIIGG	277
X. campestris pv. *phaseoli* GSA aminomutase	263 TTFGKIIGG	271
P. freudenreichii GSA aminomutase	266 FTFGKVIGG	274

FIG. 5.2 The conserved amino acid sequences surrounding the putative pyridoxal binding lysine.

生物間におけるALA生合成経路の分布をまとめたものを次のFIG.5.3に示す。これはWooseらによって分類された系統樹（Woose. C. R.(1987) Microbiol. Rev. 51,221-271)に、現在までに報告されたALA生合成経路を重ね合わせたものである。植物以外の真核生物においてはC4経路が、植物、藻類、古細菌、真性細菌においてはC5経路が確認されている。本研究で材料としたE. coli、X. campestris pv. phaseoliはpurple bacteria のγ-subgroupに、P. freudenreichiiはgram-positive bacteriaにそれぞれ分類されておりこれまでの報告と一致した(Avissar. Y. J., J. G. Ormerod, S. I. Beale (1989) Arch. Microbiol. 151, 513-519)。

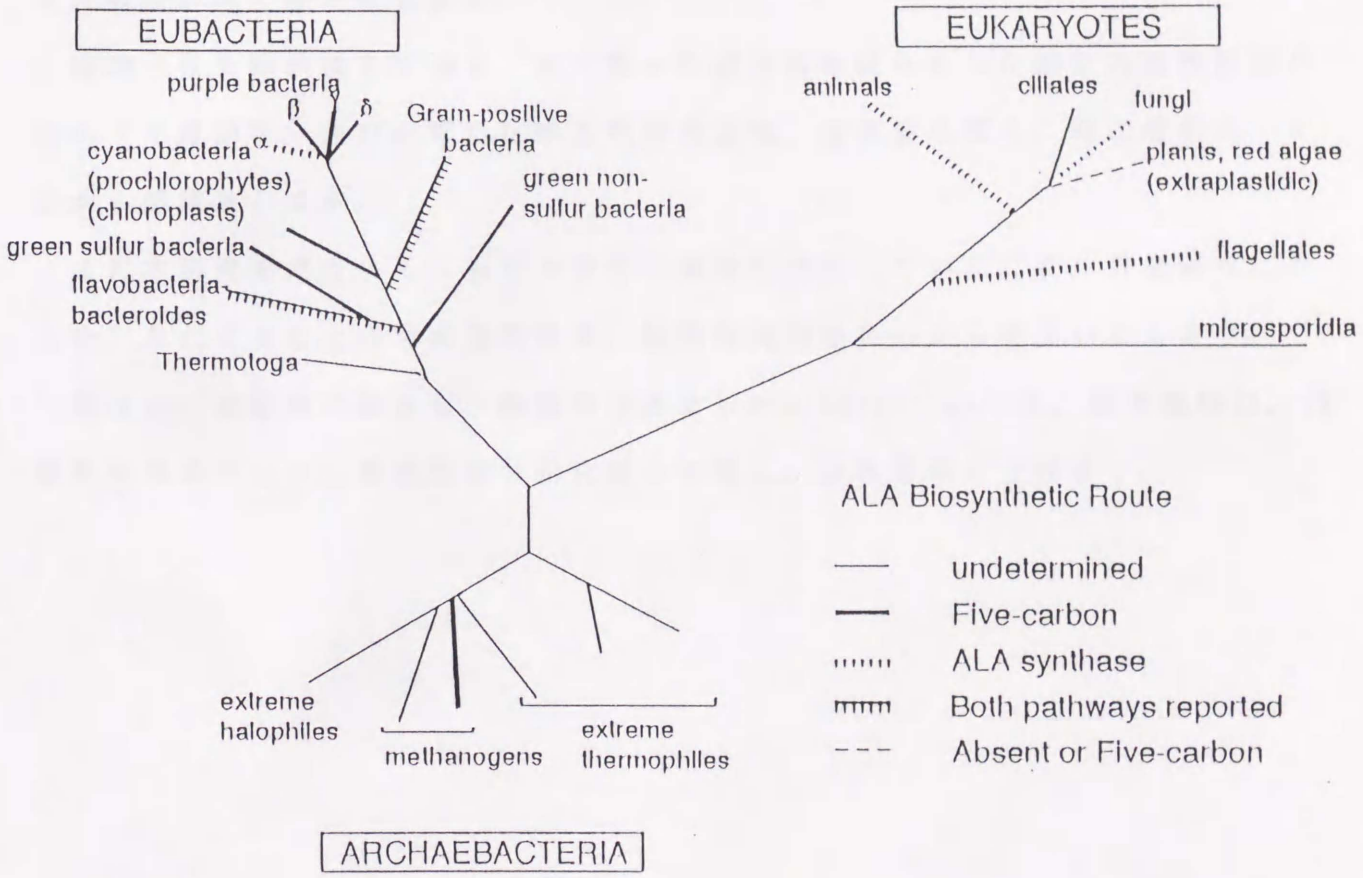

FIG. 5.3 Universal phylogenetic tree illustrating the distribution of the two ALA biosynthetic routes.

以上の結果より考察すると、細菌におけるALA生合成経路についてC4経路とC5経路のどちらか、もしくは両者の共存が考えられているが、大腸菌においてはこれら２つ以外の別経路が示唆されたことから、これら以外にもALA生合成経路が存在し、細胞にとって必須なALAの生合成、その調節が行われているものと考えられた。

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