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Genetic Controls of Sulfate and Monoamine Regulons in Enteric Bacteria

（腸内細菌のサルフェートおよびモノアミンレギュロンの遺伝子調節機構）

1994

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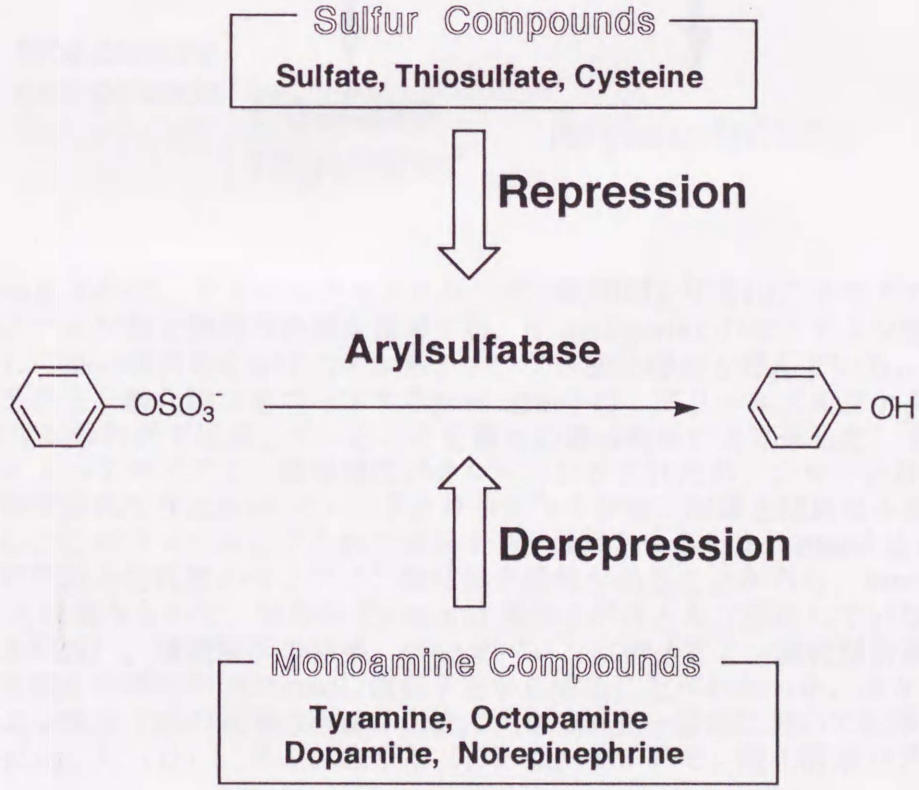
緒　言

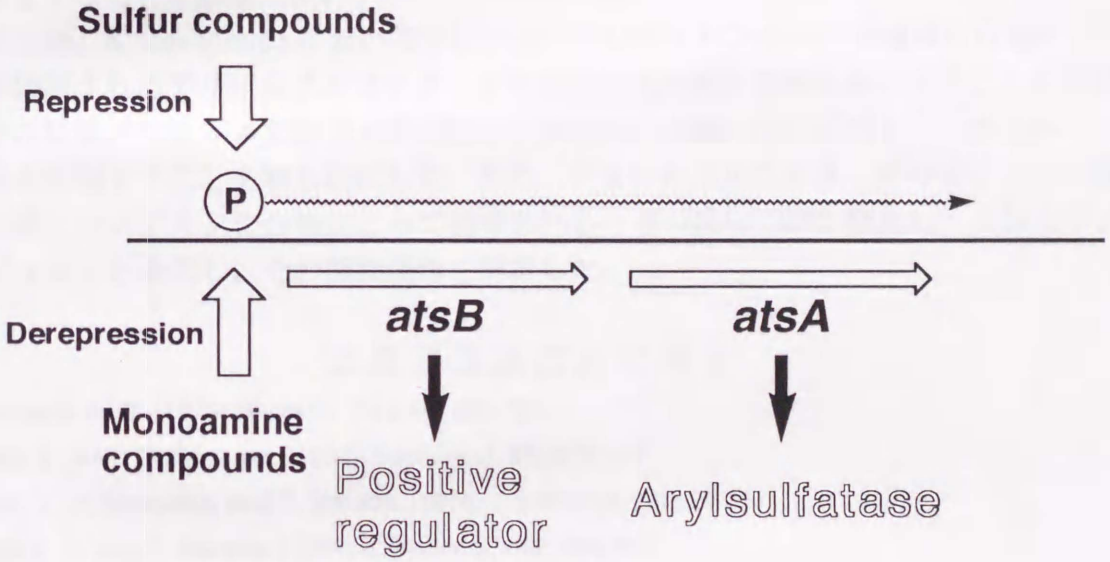
アリールスルファターゼは、アリール硫酸エステルを加水分解する酵素で、動植物組織、微生物界に広く分布し、生体内および地球上の硫黄化合物やアリール化合物の循環代謝に重要な働きをすると考えられている（1）。しかしながら、その生体内での機能についてはあまり分かっていない。　バクテリアのアリールスルファターゼ合成は、硫黄化合物とモノアミン化合物によって調節を受けることから（2）非常に興味がもたれ、*Escherichia coli*（3）や*Salmonella typhimurium*（4）にはほとんど活性がないことから、その生合成調節に関する研究は*Klebsiella aerogenes* において行われてきた。

K. aerogenes のアリールスルファターゼは分子量47000のモノマー蛋白質で（5、6）、細胞のペリプラズムに位置している（7）。*K. aerogenes*のアリールスルファターゼ合成は、無機硫酸やチオ硫酸、システインなどの硫黄化合物によって抑制されるが、メチオニンやタウリンを単一硫黄源として培養したときには抑制されず、構成的に行われる（2、3）。しかしながら、この酵素合成の硫黄化合物による抑制は、一見全く無関係と思われるチラミンやドーパミン、オクトパミン、ノルエピネフィリンなどのモノアミン化合物によって脱抑制される（8、9）。これらの化合物は、いずれも脳神経細胞において神経伝達系の調節に関与することが分かっており、腸内細菌におけるそれらの役割を明らかにすることは非常に興味深い。また、*K. aerogenes* のアリールスルファターゼをイオン交換やアフィニティーのクロマトグラフィーを用いて精製したところ、メチオニンを硫黄源にした時に生産される構成的酵素もチラミンによって脱抑制された酵素も同一のものである（6）ことが分かり、このことは遺伝解析の結果とも一致した。精製された酵素は*m*-,*o*-, *p* -nitrophenyl sulfateや*p* -nitro catecohl sulfateに高い基質特異性を示し、tyrosyl-*o* -sulfate、tyramine-*o* -sulfateやindoxyl sulfateに対しても基質特異性ををもっていた。

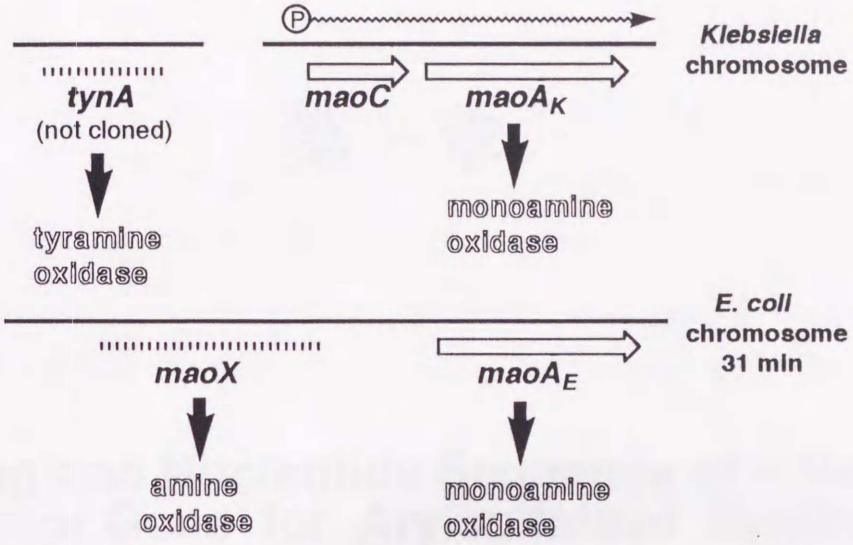


以前、足立ら（2）によって無機硫酸からシステインに至る合成系の変異株を分離したところ、システインへの合成系が止められても無機硫酸によるアリールスルファターゼの抑制がみられた。このことから、無機硫酸とシステインは別々にコレプレッサーとして働き、*atsR* でコードされているレプレッサー蛋白を活性化していると思われた。また彼らは硫黄源の種類に関係なくアリールスルファターゼを構成的に合成するAtsR変異株を分離した（3）。PW52ファージを用いた遺伝解析の結果、この*atsR* 変異はアリールスルファターゼの構造遺伝子をコードする*atsA* 遺伝子に隣接することが示唆された（10）が、後に著者らの研究により、AtsR変異は抑制遺伝子*atsR* の変異ではなく、構造遺伝子*atsA* 上流の調節領域の変異であることがわかった（未発表）。

また、最近室岡ら（5）によってアリールスルファターゼの構造遺伝子がクローニングされ、酵素合成に必須な領域のＤＮＡ塩基配列を決定した。転写産物の解析の結果、*ats* オペロンはアリールスルファターゼ遺伝子、*atsA* と、その正の調節因子*atsB* とから成ることがわかった。そして、アリールスルファターゼの硫黄化合物による抑制とモノアミン化合物による脱抑制は*atsB* 上流のプロモーター領域において転写レベルで行われていることを明らかにした。



K. aerogenes において、アリールスルファターゼの脱抑制を引き起こすモノアミン化合物は同時にモノアミン酸化酵素の合成を誘導する。*K. aerogenes* のモノアミン酸化酵素はチラミンに対して高い特異性を示すことから、チラミン酸化酵素と呼んでいる。以前室岡ら（11）は、チラミン酸化酵素をコードする*tynA* 遺伝子は、アリールスルファターゼ遺伝子*atsA* と染色体26分付近で近接していることを種々の遺伝解析により示した。また近年、杉野ら（12）によってモノアミン酸化酵素がクローニングされたが、クローン化された酵素はこれまで研究されてきた*tynA* でコードされるチラミン酸化酵素とは異なる基質特異性を示すことから、このクローニングされた遺伝子を*maoA* と命名した。*maoA* を遺伝子破壊した株にも野性株と同程度のモノアミン酸化酵素活性があることからも、*maoA* 遺伝子が*tynA* 遺伝子とは違うもので、染色体上の*maoA* 遺伝子がほとんど機能していないことが示唆された（未発表）。構造解析の結果、*mao* オペロンはモノアミン酸化酵素遺伝子*maoA* とその上流に位置する機能不明の*maoC* 遺伝子とから成ることがわかった。また、モノアミン化合物による酵素合成の誘導は*maoC* 上流のプロモーター領域において転写レベルで行われることを示した（12）。また、山下ら（13）は、モノアミン酸化酵素のアミノ酸配



列中にトーパキノンに変換し得るチロシン残基を含むコンセンサス配列を見つけ、さらに銅を補酵素とすることを示唆した。

本研究では、*K. aerogenes* においてサルフェートレギュロンの一つで硫黄化合物によりその合成が抑制されるアリールスルファターゼの遺伝子発現調節機構を解明することを目的とした。その結果、ジヒドロ葉酸還元酵素遺伝子が硫黄化合物の存在下でアリールスルファターゼ合成を抑制することを明らかにした。また、アリールスルファターゼやモノアミン酸化酵素と同様にモノアミン化合物によって誘導される一連の遺伝子群を発見し、これをモノアミンレギュロンと命名し、その調節機構を解析した。

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第一章

Cloning and Nucleotide Sequence of a Negative Regulator Gene for Arylsulfatase Synthesis in *Klebsiella aerogenes*, That Is Proved to be the *folA* Gene

ABSTRACT

A negative regulator gene for synthesis of arylsulfatase in Klebsiella aerogenes was cloned. Deletion analysis showed that the regulator gene was located within a 1.6-kilobase cloned segment. Transfer of the plasmid, which contains the cloned fragment, into constitutive atsR mutant strains of K. aerogenes resulted in complementation of atsR; the synthesis of arylsulfatase was repressed in the presence of inorganic sulfate or cysteine and this repression was relieved, in each case, by the addition of tyramine. The nucleotide sequence of the 1.6-kilobase fragment was determined. From the amino acid sequence deduced from the DNA sequence, we found two open reading frames. One of them lacked the N-terminal region but was highly homologous to the gene which codes diadenosine tetraphosphatase (apaH) of Escherichia coli. The other open reading frame was located counterclockwise to the apaH-like gene. This gene was highly homologous to the gene which codes dihydrofolate reductase (folA) of E. coli. We detected 30 times more higher activity of dihydrofolate reductase in the K. aerogenes strains carrying the plasmid, which contains the arylsulfatase regulator gene, than the strains without plasmid. Further deletion analysis showed that the K. aerogenes folA gene is consistent with the essential region required for the repression of arylsulfatase synthesis. Transfer of a plasmid containing the E. coli folA gene into atsR mutant cells of K. aerogenes resulted in repression of the arylsulfatase synthesis. Thus, we conclude that the folA gene codes a negative regulator for the ats operon.

INTRODUCTION

Arylsulfatase is involved in the metabolism of sulfur and aryl compounds; it hydrolyzes arylsulfate esters to aryl compounds and inorganic sulfate (7). Synthesis of arylsulfatase in bacteria is of interest because it is controlled by sulfur compounds and by an aromatic monoamine compounds. Most studies on the regulation of arylsulfatase biosynthesis have been performed with Klebsiella aerogenes because the enzyme is absent or a very low level from most strains of Escherichia coli (2, 10) and absent from most strains of Salmonella typhimurium (18). Arylsulfatase of K. aerogenes is a monomer, with a molecular weight of 47,000 (17, 21) and located in the periplasmic space of the cell (19). The synthesis of the enzyme is repressed by sulfur compounds, such as sulfate, thiosulfate, and cysteine, whereas the enzyme is synthesized constitutively in cells grown with methionine, or taurine as the sole source of sulfur (2, 10). Adachi et al. (2) isolated mutant strains of K. aerogenes, which are defective in the synthetic pathway from sulfate to cysteine. However, in these atsC-strains, wild type patterns of repression of arylsulfatase by sulfate or cysteine persist. These results suggest that there are two independent functional corepressors of arylsulfatase synthesis. They also isolated arylsulfatase constitutive mutant strains (2). In these AtsR$^-$ mutants, the enzyme was synthesized constitutively regardless of the source of sulfur (2, 16). These results suggested that sulfate and cysteine acted as the corepressor respectively, and that the gene coding arylsulfatase, atsA, was repressed by the atsR gene in the presence of these corepressor. The atsR mutations were more than 90% cotrans-

ducible with atsA mutations by PW52 bacteriophage transduction (5).

The repression caused by the sulfur containing compounds is relieved by the addition of tyramine, octopamine, dopamine, or norepinephrine (3, 20). These monoamine compounds induced monoamine oxidase, maoA (26, previously called tyramine oxidase, tynA) in K. aerogenes (20, 21). The derepression of arylsulfatase synthesis and the synthesis of monoamine oxidase occurred coordinately. The genetic mapping of the atsA and maoA genes on the chromosome of K. aerogenes were accomplished by using F' episomes from E. coli and the transducing phage P1. These genes are linked to gdhD and trp genes in order atsR-atsA-maoA-gdhD-trp (19). Recently, the atsA and maoA genes have been cloned, and the nucleotide sequences of regions essential for the expression of arylsulfatase (17) and monoamine oxidase (Sugino et al. in preparation) have been determined. From the analysis of the transcripts, we found that the ats operon is composed of the arylsulfatase (atsA) gene and the positive regulator gene, atsB (17). However, the molecular mechanism of the repression of the atsA gene by sulfur-containing compounds has not yet been clarified.

This report describes the cloning, characterization, and nucleotide sequence of a negative regulator gene for arylsulfatase from K. aerogenes, and we found a very interest fact that the negative regulator gene codes dihydrofolate reductase (folA).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Plasmids. pKI212, a 5.5-kilobase (kb) plasmid which confers resistance to kanamycin (Km^r), was constructed by inserting a 1.1-kb PstI fragment containing the Km^r gene from pUC4K in the PstI site of the plasmid pBR322 (5). Unless otherwise mentioned, other recombinant plasmids were derivatives of plasmid pKI212. Clone 4A3 containing the region of dihydrofolate reductase gene of *E. coli* was provided by Y. Kohara (National Institute of Genetics, Mishima, Japan).

Enzymes and chemicals. Restriction endonuclease, T4 DNA ligase, and calf intestine alkaline phosphatase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) or Toyobo Co., Ltd. (Osaka, Japan). The M13 sequencing kit (Sequenase, version 2.0, 7-deaza-dGTP edition) was provided by U. S. Biochemical Co. (Cleveland, OH). [α-^{32}P]dCTP (>650 Ci/mmol) was purchased from ICN Biomedical, Inc. (Irvine, CA). Indoxylsulfate and p-nitrophenylsulfate were provided by Sigma Chemical Co., (St. Louis, MO). The other compounds used were standard commercial preparation.

Culture media. The rich media used were LB (1% polypeptone, 0.5% yeast extract, 0.5% NaCl). The minimal media used for *K. aerogenes* were K medium (16), consisting of 0.5% carbon source, 0.1% nitrogen source, 0.01% $MgCl_2.6H_2O$, 0.001% each of NaCl, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$ and 0.05M potassium phosphate buffer

(pH7.2). The media used for E. coli strains were supplemented with 1 mM $CaCl_2.2H_2O$, threonine and leucine (20 μg/ml) and thiamine (10 μg/ml). Unless otherwise mentioned, xylose for K. aerogenes and succinate for E. coli were used as carbon, and NH_4Cl and Na_2SO_4 were used as nitrogen and sulfur sources, respectively.

Manipulation of DNA. Preparation of plasmid and phage DNA, restriction endonuclease digestion, ligation, and agarose gel electrophoresis were performed according to the methods of Maniatis et al. (13). Transformation of E. coli strains was performed according to the methods of Hanahan et al. (9). For transformation of K. aerogenes cells, a modification of the method as described by Davis et al. (6) was used. The cells were treated with 100mM $CaCl_2$, 50mM RbCl, and 25mM LiCl in 100mM 3-(N-morpholino) propanesulfonate (pH 6.5). After incubation for 1 hr at 0° C, the cells were heated at 42° C for 2 min and then washed with saline twice to remove the excess metal ions.

Cloning of the gene complemented to atsR mutations. Chromosomal DNA prepared from K. aerogenes W70 by the method of Marmur (14) was partially digested with EcoRI or BamHI. Fragments of between 3 and 10 kb of the chromosomal DNA were isolated by sucrose gradient centrifugation and ligated to EcoRI- or BamHI-cleaved pKI212 by T4 DNA ligase after treatment with alkaline phosphatase. Arylsulfatase constitutive mutant atsR cells were transformed by the $CaCl_2$-heat shock method as described above. Transformants were selected on LB agar plates which contained 50 μg/ml of kanamycin and Km^r colonies were replicated onto K medium with 0.25 mg/ml of indoxylsulfate as an indicator of

arylsulfatase activity (1). Colonies with low arylsulfatase activity are colorless whereas AtsR$^-$ colonies with high enzyme activity are blue owing to hydrolysis of the enzyme substrate. Transformants yielding colorless colonies were selected.

DNA sequence analysis. DNA sequence was determined by the M13-dideoxynucleotide chain-termination method (22). In all cases, sequencing reactions were performed with T7 polymerase kits including [α-^{32}P] dCTP as the DNA labeling and deoxy-7-deazaguanosine triphosphate instead of deoxy-guanosine triphosphate to eliminate the G-C band compression (15). The sequencing reaction products were resolved on 6 % polyacrylamide-8 M urea sequencing gels. Gels were run at 2000V and between 50° C and 55° C, dried on a Whatman 3MM filter paper (Whatman, Inc., Clifton, NJ), and visualized by autograph using X-OMAT AR (Eastman Kodak Co., Rochester, NY) film.

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

Assay of enzyme activities. Bacteria were grown aerobically at 28° C in K medium. Tyramine was used as an inducer of the derepressed synthesis of arylsulfatase. Growth was monitored with a Klett-Summerson colorimeter. Arylsulfatase activity was assayed as described previously (1). One unit of arylsulfatase was defined as the amount of enzyme causing formation of one nmol of p-nitrophenol per min at 30° C.

Assay of K. aerogenes dihydrofolate reductase was based on that of Stone and Morrison (24, 25) and performed as follows.

Bacteria were grown aerobically at 28°C in K medium. When the culture reached 100-200 Klett units, cells from a 1 ml portion of the culture were harvested and suspended in 300 μl of 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. Crude extract (5-50 μl) was added to 2.5 ml of 50 mM Tris-HCl (pH7.4) containing 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM NADPH. One ml aliquots of the mixture were added to each of two cuvettes. Dihydrofolate was added to 20 μM in the sample cuvette, and an equal volume of buffer was added to the reference cuvette. The change in absorbance at 340 nm was followed using a JASCO UVIDEC-340 (JAPAN SPECTROSCOPIC Co.,Ltd., Tokyo, Japan).

Hybridization for gene mapping. The membrane that was used was Gene Mapping Membrane (Takara Shuzo Co., Ltd., Kyoto, Japan), on which immobilized cloned phages covering E. coli genome developed by Kohara et al. (11) were loaded. Hybridization of the material on the membrane with the DNA probe, which was labeled with nonradioactive DNA labeling and detection kit of Boehringer Mannheim Biochemica (Mannheim, Germany), was performed according to the recommendations of the manufacturer.

RESULTS

Cloning of the gene complemented *atsR* mutation. Chromosomal DNA from *K. aerogenes* W70 was partially digested with *Eco*RI or *Bam*HI. The fragments were ligated with *Eco*RI- or *Bam*HI-cleaved pKI212. The mixture of hybrid DNA molecules was used to transform *K. aerogenes* K304, which is the *atsR* mutant strain and permits constitutive synthesis of arylsulfatase in the presence of sulfate. Among about 10,000 Km^r colonies tested, one colorless colony was obtained. We will designate this as functionally $AtsR^+$ since it is not stained blue in the presence of indoxylsulfate. Plasmid DNA prepared from this strain was about 5.8 kb in length, and this plasmid was designated pASR001.

The transformation of *K. aerogenes* K304 (*atsR4*) with pASR001 yielded 100% Km^r, $AtsR^+$ colonies, indicating that the recombinant plasmid was responsible for the ability to repress the synthesis of arylsulfatase. The pASR001 plasmid was further used to transform another *atsR* mutant, *K. aerogenes* K311 (*atsR11*), as well as other unpublished mutants with different *atsR* alleles: all transformants were colorless on indoxylsulfate plates.

Effect of pASR001 on the expression of *atsA*. Effect of the cloned fragment on the plasmid pAS001 on the synthesis of arylsulfatase in cells of constitutive *atsR* mutant strains, *K. aerogenes* K304 and K311, was tested under various conditions. Arylsulfatase activities in the cells grown in synthetic media with inorganic sulfate, cysteine, or methionine as the sole source of sulfur with and without tyramine were observed (Table 2).

In *K. aerogenes* W70, the synthesis of arylsulfatase was

repressed when the cells were grown with inorganic sulfate or cysteine, whereas the enzyme was synthesized when methionine was used as the sole source of sulfur as shown previously (2). The repression of the enzyme by these sulfur compounds was relieved by the addition of tyramine. In the atsR mutant strains, K304 and K311, levels of arylsulfatase were rather high and synthesis of arylsulfatase was not repressed even when inorganic sulfate or cysteine was present. Addition of tyramine to strains K304 and K311 did not appreciably stimulate the already constitutive levels of enzyme activity except when methionine was present as the sole source of sulfur and for strain K311, when Na_2SO_4 was the sulfur source (Table 2). When plasmid pASR001 was present in these mutant strains, arylsulfatase activity was lowered 5- to 10-fold, but not to basal activity levels present in the wild-type strain without the plasmid. Repression by pASR001 was also generally relieved by the addition of tyramine, although the derepression level in strain K304 was low (Table 2). Thus, pASR001 lowers the constitutive levels of arylsulfatase in atsR mutants and therefore pASR001 appears to give at least partial complementation of the atsR mutation.

Localization of the regulator gene. A restriction map of the cloned chromosomal fragment was constructed (Fig. 1). A 5.8-kb chromosomal fragment on the pASR001 plasmid was digested with EcoRI and BamHI and subcloned into the pKI212 vector. Two plasmids, pASRB1 and pASRB2, which contained the 4.2-kb EcoRI-BamHI fragment and 1.6-kb BamHI-EcoRI fragment, respectively, were constructed. The properties of the plasmids were examined by

analyzing the complementation with the atsR mutation. The pASRB2 repressed the arylsulfatase synthesis in strain K304. These results suggest that a negative regulator gene for atsA expression is located within the 1.6-kb EcoRI -BamHI fragment (Fig. 1).

Nucleotide sequence of the negative regulator gene. The complete nucleotide sequence of the 1.6-kb EcoRI-BamHI fragment, which carries the regulator gene was determined (Fig. 2). We found two open reading frames (ORFs) capable of coding for about 18- and >29-kilodalton proteins. The first ORF consists of 480 bp, with a putative ATG initiation codon at position 145 and a TAA termination codon at position 622. The putative initiation codon is preceded by a sequence with a high degree of similarity to the -10 and -35 consensus sequence (TcGACg-17bp-TATAgT) of E. coli and has a potential ribosome-binding site (gGGAa) (23). In the 3'-flanking region of this ORF, we found a palindromic sequence (ΔG; -37.7 kcal [ca. -158 KJ]/mol) which may act as a bidirectional ρ-independent transcription terminator. The second ORF seemed to lack the N-terminal amino acid sequence and contains >780 bp with a TGA termination codon at position 877. This ORF was located counterclockwise to the first ORF.

Homology search. The amino acid sequence deduced from the DNA sequence indicates that the product of the first ORF contains amino acids residues, having a molecular weight of 18,139. We have searched for homologies in the DNA and peptide sequences of the ORFs and other genes with EMBL and SWISS data bases. We found that the sequences of the first ORF are 80.0% homologous to the sequence of DNA and 90.6% homologous to the sequence of amino acid from the sequences of the E. coli dihydrofolate reductase

gene, folA, respectively (Fig. 3). In addition, we found that the second ORF (C-terminal portion) is located counterclockwise to the folA-like gene (Fig. 3), and sequences of the DNA and amino acid are 79.1 % and 87.6 % homologous to those from the E. coli diadenosine tetraphosphatase (apaH), respectively. From comparison of the amino acid sequence, we found that 20 amino acids from the N-terminus of the K. aerogenes apaH were probably lacking in our clone.

Determination of the essential region for the atsA repression. We further subcloned a variety of deletion plasmids to determine the region responsible for lowering of arylsulfatase activity (Fig. 4). Properties of these plasmids were examined by performing complementation tests with the atsR strains of K. aerogenes. The deletion analysis suggests that the regulator gene is located within the 760 bp Eco47III-BamHI fragment. Since this region was coincident with the ORF of the folA-like gene, the folA-like gene seemed to repress the arylsulfatase synthesis.

Estimation of dihydrofolate reductase activity. Activities of dihydrofolate reductase in the wild-type and atsR strains with and without plasmid pASRB2 were measured. K. aerogenes strains carried pASRB2 showed about 20- to 30-fold higher activities of dihydrofolate reductase than those of the strains without plasmid (Table 3). This result indicates that the folA-like gene codes for functional dihydrofolate reductase. Thus, we concluded that the first ORF is the folA gene of K. aerogenes. However, dihydrofolate reductase activities in atsR mutant stains were similar to that of the wild-type strain.

Analogous mapping of the folA gene on the E. coli chromosome. By using the clone of the K. aerogenes folA gene ($folA_K$), the analogous location of the folA gene in E. coli was mapped. A recently developed physical map of the E. coli W3110 chromosome generated from overlapping λ phage clones encompassing the entire E. coli genome was used (11). A 0.68-kb SmaI-SalI fragment was isolated from pASRB2 and labeled with non-radioactive DNA labeling kit. The $folA_K$ probe hybridized strongly to the coordinate positions of clone 4A3 on the gene mapping membrane that is located at 1 min of E. coli genome (Fig. 5). This result is consistent with the previous mapping of the folA gene together with the apaH gene of E. coli genome (4).

Repression of K. aerogenes atsA expression by E. coli folA. Next, we tested whether the E. coli folA gene could complement the K. aerogenes atsR mutation. The folA region of E. coli was cut out with EcoRV from the clone 4A3 and the fragment was inserted into pKI212 vector. The resultant plasmid was designated pEFOL2. The plasmid pEFOL2 was introduced into the atsR mutant strains of K. aerogenes. The plasmid pEFOL2 repressed the arylsulfatase synthesis in the presence of inorganic sulfate (Table 3). The repression level by the $folA_K$ of E. coli was the same as that by the K. aerogenes folA.

From these results, we conclude that the folA genes from either K. aerogenes or E. coli represses otherwise constitutive atsA expression in atsR mutants.

DISCUSSION

We have cloned a chromosomal fragment of K. aerogenes W70, which in multicopy apparently repress arylsulfatase synthesis of atsR mutants in the presence of sulfur compounds. Thus, the transfer of the recombinant plasmid into the atsR-deficient mutant strains resulted in complementation of the atsR mutation. Deletion analysis of the plasmid showed that all of the sequences required for the repression of the arylsulfatase synthesis are located within a 0.76-kb segment of DNA (Fig. 4). This segment contained the ORF which possesses DNA and amino acid sequences strongly homologous to the folA gene of E. coli. Although this newly found regulator gene in multicopy repressed the arylsulfatase synthesis of atsR mutant strains, the repressed levels in these strains were not as low as that of wild-type strain W70. Furthermore, the folA gene in multicopy did not appreciably further repress the wild type levels of AtsA expression for cells grown on sulfate or cysteine as sole sources of sulfate (Table 2). These results suggest that another negative factor might be required for the complete repression of arylsulfatase synthesis in the presence of sulfur compounds in atsR mutants. Alternatively, a low efficiency of simple reversal of atsR-mediated constitutive expression of atsA may occur.

All results presented here, that is, deletion analysis of the regulator gene caused atsA repression, sequence homologies of DNA and amino acid of the regulator to those of E. coli folA, over production of dihydrofolate reductase by the K. aerogenes carrying plasmid with the regulator gene, and the complementation of

the E. coli folA gene to the K. aerogenes atsR mutation, show that the newly cloned negative regulator gene for arylsulfatase synthesis is the folA gene. Like the regulation pattern of arylsulfatase in K. aerogenes wild-type strain (16), the repression of constitutive atsR mutants by the $folA_K$ gene was repressed by sulfur, and with the exception of the atsR4 mutant grown on cysteine, this repression was generally relieved by tyramine (Table 2). These results suggest that the repression by the folA gene is involved in a negative regulation system for the ats operon in K. aerogenes.

Previously, Murooka et al. (16) showed that an E. coli gene analogous to the atsA gene of K. aerogenes is located at 27 min on the E. coli genome by classical genetic analysis. The E. coli gene is judged as analogous to the Klebsiella atsA gene because of common regulatory features discovered lac operon fusions (28). By the physical mapping of the $folA_K$ with Kohara bank of E. coli genome, the $folA_E$ gene is located at 1 min on the E. coli chromosome, far from the location the E. coli atsA homolog just mentioned. Previously transduction experiments showed that atsR is more than 90% linked with the atsA gene (16). This result indicates that the product of the folA gene is different from the predicted atsR aporepressor. Arylsulfatase activity is not demonstrable in E. coli, but a protein was found that is cross-reactive with anti-AtsA antibody made to the Klebsiella AtsA protein (27). The shared ability of the E. coli folA gene ($folA_E$) and $folA_K$ to repress atsA expression in K. aerogenes strongly suggests that the Klebsiella regulatory mechanism is conserved in E. coli.

Since no significant differences of dihydrofolate reductase activities were found between wild-type strain W70 and the atsR mutant strains in the absence of plasmids, the atsR mutation does not result in constitutive levels of AtsA through effects on single copy folA gene expression. Elucidation of the role of the repression mechanism of atsA expression by dihydrofolate reductase, which is a key enzyme in folic acid metabolism (8), may involve direct effects on atsA expression or metabolic effects on enzyme activity. Alternatively, it may involve effects acting through the positive regulator encoded by atsB either at the level of gene expression or enzyme activity. In any case, the significance of the work presented here is that it implies the existence of an unexpected regulatory connection between methyl (C1) metabolism and sulfur metabolism.

ACKNOWLEDGMENTS

We thank Y. Kohara and A. Ishihama of National Institute of Genetics, Japan for providing E. coli genomic mapping membrane and E. coli genomic clones and to M. Cashel of National Institutes of Health, USA for his helpful information and critical comments on the manuscript.

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TABLE 1. List of bacterial strains and their characteristics

Strain	Relevant genotype	Source or reference
K. aerogenes		
W70	Wild-type	MacPhee et al. (12)
K304	*tynA13 atsR4*	Murooka et al. (16)
K311	*tynB13 atsR11*	Murooka et al. (16)
E. coli		
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44*, λ^-, Δ(*lac-proAB*), *relA1*, [F', *proAB*, *lacIqZ* M15, *traD36*]	Yanisch-Perron et al. (29)

TABLE 2. Effect of the pASR001 on the repression of arylsulfatase synthesis *K. aerogenes* strains[a]

Host strain (genotype)	Plasmid (pASR001)	Sulfur source (3 mM)	Tyramine (3 mM)	Arylsulfatase activity (U/mg of cells)
W70 (wild-type)	-	Na_2SO_4	-	0.27
	-	Na_2SO_4	+	3.58
	-	Cysteine	-	0.30
	-	Cysteine	+	3.58
	-	Methionine	-	2.27
	-	Methionine	+	9.96
	+	Na_2SO_4	-	0.22
	+	Na_2SO_4	+	2.00
	+	Cysteine	-	0.24
	+	Cysteine	+	2.10
	+	Methionine	-	0.71
	+	Methionine	+	5.45
K304 (*atsR4*)	-	Na_2SO_4	-	11.6
	-	Na_2SO_4	+	12.0
	-	Cysteine	-	11.6
	-	Cysteine	+	11.6
	-	Methionine	-	5.52
	-	Methionine	+	7.64
	+	Na_2SO_4	-	2.08
	+	Na_2SO_4	+	5.07
	+	Cysteine	-	2.32
	+	Cysteine	+	2.50
	+	Methionine	-	2.86
	+	Methionine	+	3.56
K311 (*atsR11*)	-	Na_2SO_4	-	11.3
	-	Na_2SO_4	+	15.0
	-	Cysteine	-	11.2
	-	Cysteine	+	9.26
	-	Methionine	-	5.80
	-	Methionine	+	14.0
	+	Na_2SO_4	-	2.62
	+	Na_2SO_4	+	11.9
	+	Cysteine	-	1.33
	+	Cysteine	+	9.68
	+	Methionine	-	2.29
	+	Methionine	+	10.1

[a]The cells were grown in xylose-NH_4Cl medium with the source of sulfur as indicated, in the presence (+) or absence (-) of tyramine. The cells were harvested when the density of the culture had reached about 100 Klett units. Values are averages of results from three independent experiments.

TABLE 3. Activities of arylsulfatase and dihydrofolate reductase in the *K. aerogenes* wild-type and *atsR* strains with or without plasmid[a]

Host strain (Genotype)	Plasmid	Enzyme activity (U/mg of cells)	
		Arylsulfatase	Dihydrofolate reductase
W70	-	0.15	0.03
(Wild-type)	pASRB2	0.08	0.63
	pEFOL	0.10	0.52
K304 (*atsR4*)	-	10.4	0.03
	pASRB2	1.60	0.97
	pEFOL2	1.76	0.71
K311 (*atsR11*)	-	12.1	0.03
	pASRB2	1.68	0.95
	pEFOL2	2.16	0.58

[a]The cells were grown in K medium with 3 mM sodium sulfate in the absence of tyramine. The cells were harvested and assayed when the density of the culture had reached about 100 Klett units. Values are averages of results from three independent experiments.

Legends for figures

FIG. 1. Restriction endonuclease map of pASR001 and its derivative plasmids. Thick bar represents the region of DNA cloned; thin bar represents DNA remaining in plasmid subclones. Deletion plasmids were used to transform K. aerogenes K304 and K311, and cells were assayed for arylsulfatase activity (+, repression level; -, constitutive level).

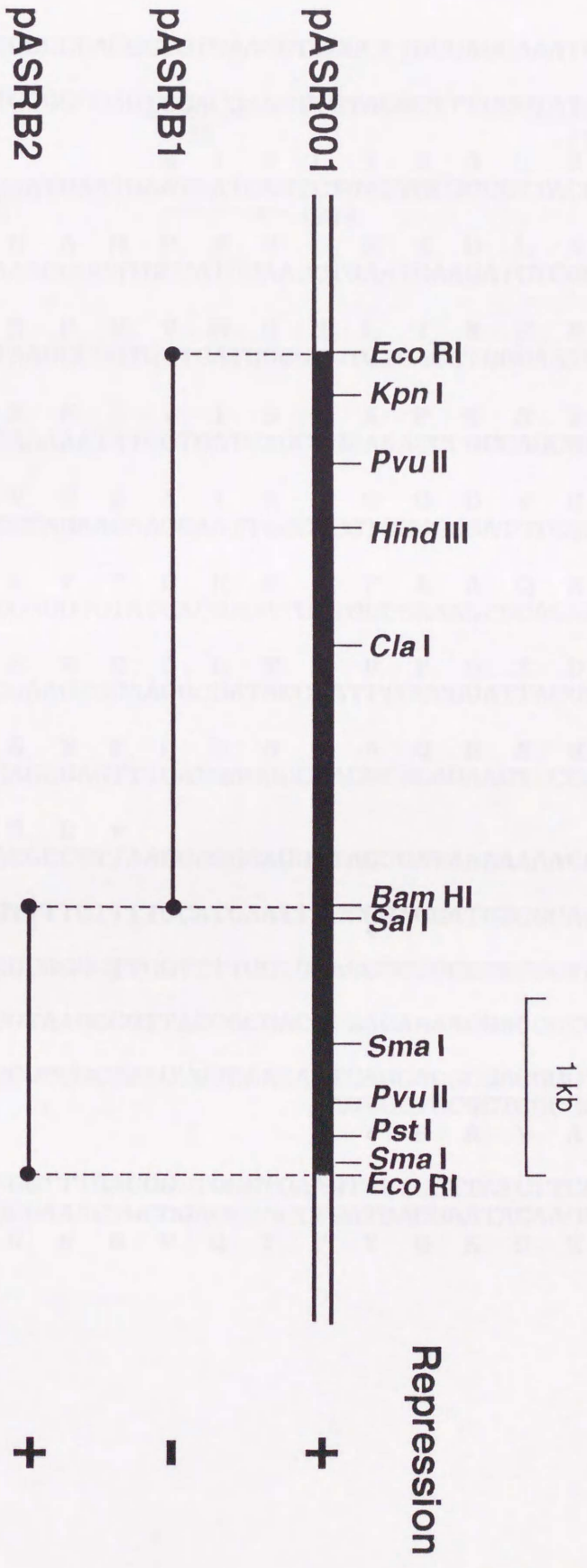
FIG. 2. Nucleotide sequence of the 1.6-kb fragment and the predicted amino acid sequences of the ORF1 and ORF2 genes. The presumptive ribosomal-binding site (SD) and possible promoter regions (-10 and -35) are indicated. The arrows show the direction of ORF1 and ORF2. The horizontal dashed arrows show inverted repeat sequences. The GenBank/EMBL accession number is ----.

FIG. 3. Comparison of folA sequences from K. aerogenes and E. coli. Identical nucleotides are indicated by shaded box with *.

FIG. 4. Restriction map of pASRB2 and its derivative plasmids. Deletion plasmids were used to transform K. aerogenes K304 and K311, and cells were assayed for arylsulfatase activity (+, repression level; -, constitutive level). The arrowhead indicates the direction of transcription of the folA and apaH genes. These genes were deduced from the sequence analysis (Fig. 3).

FIG. 5. Restriction map of E. coli clone 4A3 (11) and construction of plasmid pEFOL2. Direction of genes were determined by Blanchin-Roland et al. (4).

Fig. 1.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pASR001
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Kpn I
Pvu II
Hind III
Cla I
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Sma I
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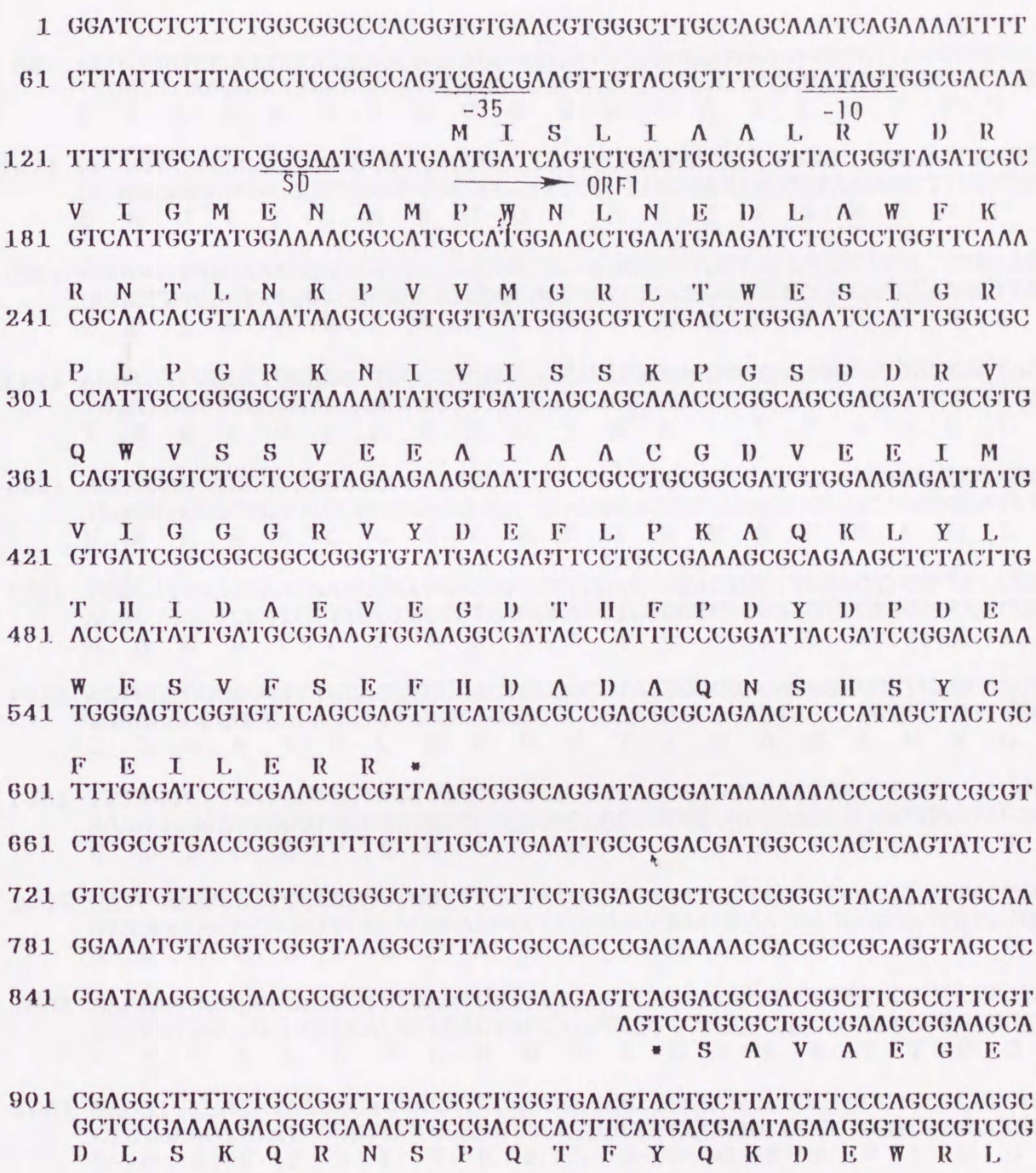
1 kb
+
-
+
Repression

Fig. 2.



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1021 CGCGTCCCTCCAGCGAAGCCCAGTGGCCAAAGGCAATGCTGTAGGCATTGCTGACCGGGC
GCGCAGGGAGGTCGCTTCGGGTCACCGGTTTCCGTTACGACATCCGTAACGACTGGCCCG
G R G E L S A W H G F A I S Y A N S V P

1081 CGGGAATGGCAAACCACGGCTTCAGCGGCGCCGGGGCGTCCTCGGGCGCTTCCTTCGAAT
GCCCTTACCGTTTGGTGCCGAAGTCGCCGCGGCCCCGCAGGAGCCCGCGAAGGAAGCTTA
G P I A F W P K L P A P A D E P A E K S

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Y M D L Q G N P F C Y R M R T F A N S I

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F R L R A L G S L E N S W H N P M D G Y

1261 TGGCATCGAGGAAGAAAGGATAGGAGTCGCTGGAGAGCACTGCTTCAACATCGCGCGCGC
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M A D L F F P Y S D S S L V A E V D R A

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C Q Q A T E L D W Q P T I G A H A M V L

1381 TTTTCTCTTCATCCACCTGCAGCAGCGGCTGGCGACGCAGCCAGTTGAGCAGCTCATCGG
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K K E E D V Q L L P Q R R L W N L L E D

1441 CATCCGGCGCTTCCAGCAGTGGTTTCAGACGATCCTTTGGCTTGTTGCGGCTGATACCGG
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A D P A E L L P K L R D K P K N R S I G

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L S K V Y R L V E L S G P G R A V L D G

1621 TCAGCCACAGCGTATCTCTCCGCGGATCGAATTC
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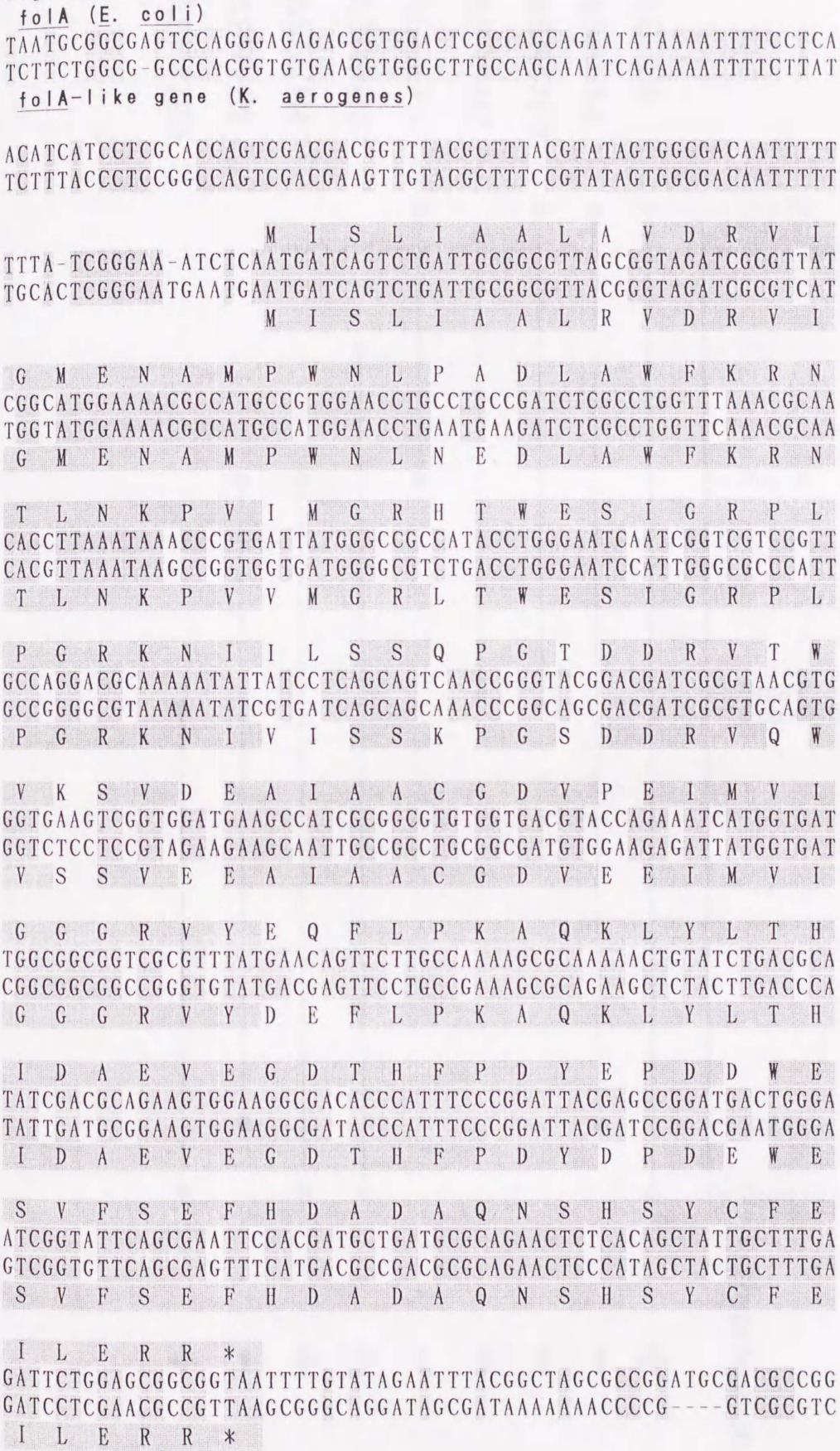
T L W L T D R R P D F E

ORF2 ←

Fig. 3.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folA-like gene (K. aerogenes)
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I L E R R *
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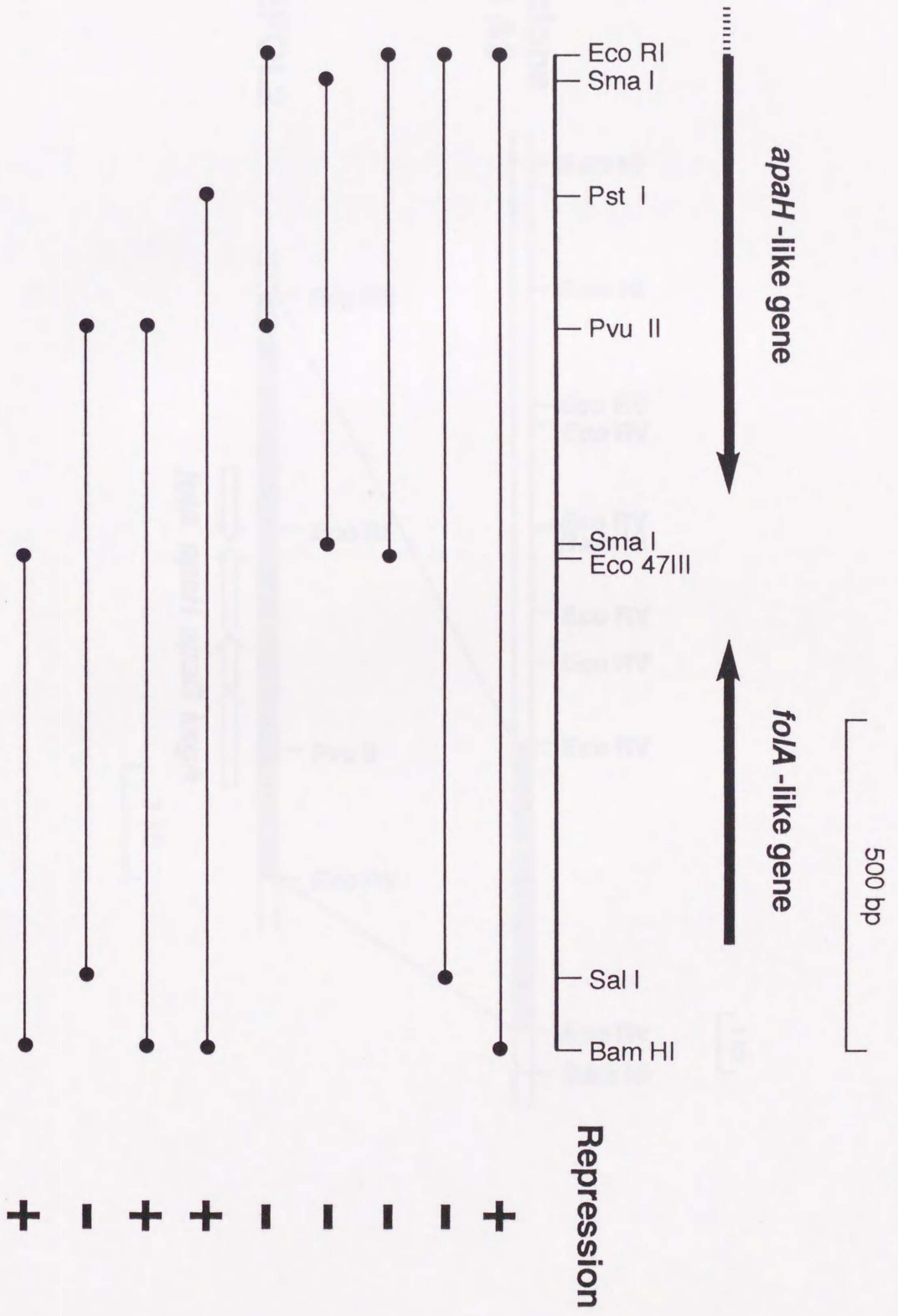
Fig. 4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Eco 47III
Sal I
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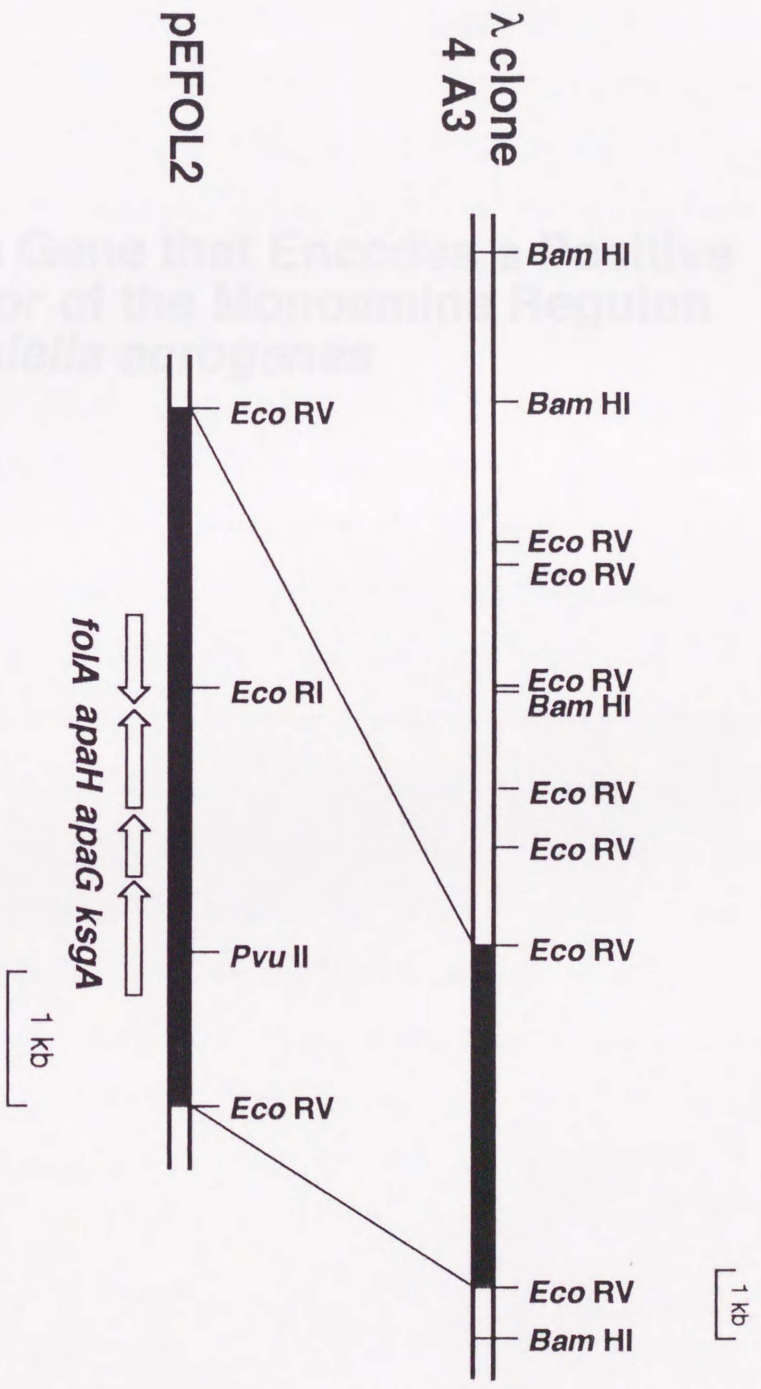
+
Repression

Fig. 5.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Pvu II
Eco RV
1 kb
Eco RV
Bam HI
1 kb

第二章

moaR, a Gene that Encodes a Positive Regulator of the Monoamine Regulon in *Klebsiella aerogenes*

ABSTRACT

We cloned and sequenced a *Klebsiella aerogenes* gene (*moaR*) for activation of arylsulfatase synthesis by tyramine. This gene was cloned by complementation of a *K. aerogenes* mutant in which tyramine fails to relieve the arylsulfatase repression caused by sulfur compounds. The *moaR* gene also activated induction of the synthesis of both tyramine oxidase and the 30-kDa protein that is specifically induced by high concentrations of tyramine or catecholamines. The *moaR* gene on the chromosome of the wild-type strain of *K. aerogenes* was disrupted by homologous recombination with a plasmid containing the inactivated *moaR*. The resultant mutant showed the same phenotype of previously isolated *atsT* mutant strains that are negative for the derepressed synthesis of arylsulfatase. In this mutant strain, tyramine also failed to induce the synthesis of tyramine oxidase or the production of a 30-kDa protein. The *moaR* gene is capable of encoding a protein of 26,238 daltons. The putative MoaR protein has a helix-turn-helix motif in its C-terminus. Thus, it seems likely that the MoaR protein regulates the operons by binding to the regulatory region of the monoamine regulon. The MoaR protein is subject to autogenous control, which was shown by use of a *moaR'-lacZ* transcriptional fusion.

INTRODUCTION

In *Klebsiella aerogenes*, arylsulfates are metabolized by an arylsulfatase (the *atsA* gene product) whose synthesis is regulated by sulfur compounds and aromatic monoamines. Synthesis of the enzyme is repressed by sulfate or cysteine but not by methionine or taurine as a sole sulfur source (2, 5, 10). In the presence of sulfate (or cysteine), arylsulfatase synthesis is induced by tyramine, octopamine, dopamine, or norepinephrine (1, 3, 10). These compounds also lead to induction of tyramine oxidase (the *tynA* gene product) and monoamine oxidase (the *maoA* gene product) (16, 21). Tyramine oxidase is more specific than monoamine oxidase for tyramine and these catecholamines (21, 27). The derepression of arylsulfatase synthesis and the tyramine oxidase synthesis occur coordinately upon the addition of these monoamine compounds (17, 21). The *atsA* gene is part of an operon with the *atsB* gene, which encodes an activator for *atsA* gene expression (19). The *maoA* gene is part of an operon with the *maoC* gene, which has unknown function (27). In addition, a gene called *atsT* was shown by Okamura et al. to be required for induction of arylsulfatase synthesis by tyramine (21).

To study the mechanism of the coordinated expression of proteins induced by monoamine compounds, we cloned and characterized a gene that is involved in the derepression of the synthesis of arylsulfatase. We found that this gene (*moaR*) plays a central role in the positive regulation of the expression of the monoamine regulon including the *ats* and *tyn* operons.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. pMC1403 (6) and pVEX11 were provided by Y. Yamada and A. Nakazawa (Yamaguchi University). pEL3 (4) was provided by E. Ohtsubo (Tokyo University). pHSG298 (28) was purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). pMW219 (29) and pUC4KIXX (30) were purchased from Nippon Gene Co., Ltd. (Toyama, Japan) and Pharmacia P-L Biochemicals, Inc. (Uppsala, Sweden), respectively.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and calf intestine alkaline phosphatase were purchased from Takara Shuzo Co., Ltd. or Toyobo Co., Ltd. (Osaka, Japan). Indoxylsulfate and p-nitrophenylsulfate were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-tyramine hydrochloride (20 Ci/mmol) and [^{35}S]-methionine was purchased from New England Nuclear Research/Du Pont (Boston, Mass.). The other compounds used were standard commercial preparations.

Culture media. The rich medium used was LB (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl). The minimal medium used was K medium (16), which consists of 0.5% xylose, 3 mM Na_2SO_4, 0.1% NH_4Cl, 0.01% $MgCl_2.6H_2O$, 0.001% each of NaCl, $MnCl_2.4H_2O$, $FeCl_3.6H_2O$ and 0.05 M potassium phosphate buffer (pH 7.2).

Manipulation of DNA. Preparation of plasmid and phage DNA, restriction endonuclease digestion, ligation, and agarose gel electrophoresis were performed by the methods of Maniatis et al. (13). Transformation of K. aerogenes was performed by the modifi-

cation (5) of the method of Hanahan (9).

Isolation of mutants. $NaNO_2$ was used as the mutagenic agent, as described by Eisenstark et al. (7). For selection of mutant strains from K. aerogenes W70, white colonies that failed to synthesize arylsulfatase in the presence of tyramine (3 mM) were selected by use of indoxylsulfate (0.25 mg/ml) as an indicator (1). Organisms were examined for the synthesis of arylsulfatase under non-repressed conditions in a liquid medium that contained methionine as the sole source of sulfur.

Cloning of the gene that complemented the moaR mutation. Chromosomal DNA prepared from K. aerogenes W70 by the method of Marmur (14) was partially digested with Sau3AI, and the fragments were ligated to BamHI-cleaved pHSG298 after treatment with alkaline phosphatase. Mutant cells that lacked the derepressed synthesis of arylsulfatase were transformed, and transformants were selected on LB agar plates that contained 50 μg/ml of kanamycin. Groups of 120 kanamycin-resistant colonies were inoculated into K medium with 3 mM tyramine and the arylsulfatase activity was measured.

Analysis of DNA sequences. Sequencing reactions were performed by the M13 dideoxy-chain termination method (23) 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 nucleotide and amino acid sequences were analyzed with GENETYX programs (SDC Software Development Co., Ltd., Tokyo, Japan). The DDBJ/GenBank/EMBL nucleotide sequence accession

number is D15072.

Gene disruption on the chromosome. A 1.2-kb SmaI fragment containing the kan gene in pUC4KIXX was inserted into an EcoRV site in the moaR gene. The inactivated moaR gene was subcloned into pEL3 (4) and resultant plasmid (pELAT5) was used to transform into K. aerogenes W70. The transformants were grown on LB plates containing kanamycin (50 μg/ml) at 42^{o}C, overnight. Since pELAT5 has the temperature-sensitive replicon, the transformants should appear as Km^{r} colonies only after homologous recombination of the plasmid (15). The insert of the kan gene into the moaR gene on the chromosome was confirmed by Southern Hybridization with non-radioactive labeling of the fragment containing moaR as a probe.

Assay of enzymatic activities. Bacterial cells were grown aerobically at 28^{o}C in K medium. The activities of arylsulfatase (1), tyramine oxidase (20) and β-galactosidase (22) were assayed as described previously. One unit of arylsulfatase was defined as the amount of enzyme causing formation of one nmol of p-nitrophenol per min at 30^{o}C. One unit of tyramine oxidase was defined as the amount metabolizing one μmol of tyramine per min at 30^{o}C. One unit of β-galactosidase was defined as the amount catalyzing the release of 1 nmol of o-nitrophenol from o-nitrophenyl-β-galactoside per min at 30^{o}C. Protein was determined by the method of Lowry et al. (11) with bovine serum albumin as a standard.

Expression of plasmid-encoded moaR under control of a T7 expression system. The 1.3-kb KpnI-HindIII fragment containing the moaR gene in pAT11 was subcloned into pVEX11 and the resultant plasmid (pVEXKH) was transformed into BL21(DE3). Ten μCi of

[^{35}S]-methionine were added to 1 ml of cells containing pVEXKH grown at 37^{o}C to an optical density of 0.5, and the suspension was shaken at 37^{o}C for 5 min (26). The concentrated cells were dissolved in 100 μl of sample buffer (pH 6.8), which contained 50 mM Tris-HCl, 2% 2-mercaptoethanol, 4% sodium dodecyl sulfate (SDS), and 5% glycerol. After the samples had been boiled for 10 min, the proteins were separated by SDS-polyacrylamide gel electrophoresis. Dried gels were overlaid with Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) for autoradiography.

RESULTS

Isolation of a mutant strain in which derepression of arylsulfatase synthesis by tyramine did not occur. We isolated a mutant strain, K. aerogenes K801, in which tyramine failed to cause the derepression of arylsulfatase synthesis, from the wild-type strain W70 in the presence of tyramine and indoxylsulfate as the inducer and indicater of arylsulfatase synthesis, respectively (Table 2). In the mutant strain K801, tyramine did not relieve the repression of arylsulfatase caused by inorganic sulfate, whereas in the wild-type strain W70 synthesis of the enzyme in the presence of sulfate was derepressed by tyramine. Since the mutant strain K801 synthesized arylsulfatase under non-repressed conditions, with methionine as the sole source of sulfur, as well as strain W70 (Table 2), the structural gene for arylsulfatase (atsA) had clearly not been mutated. Strain K801 could not utilize tyramine as the sole source of nitrogen nor did it produce any monoamine oxidases.

Cloning of the gene that complements the mutation responsible for the absence of the derepressed synthesis of arylsulfatase. A plasmid carrying the moaR gene was found by transformation of K. aerogenes K801 with a Sau3AI library and screening transformants for synthesis of arylsulfatase in the presence of sulfate and tyramine. One positive colony was found among 14,000. This plasmid (pAT11) contained a 1.3-kb insert and complemented the K. aerogenes atsT mutants, K611 and MKN204. Like strain K801, these mutants failed to synthesize arylsulfatase in the presence of tyramine and synthesized arylsulfatase during growth on methionine (16) (Table 2).

Characterization of the cloned gene. The effect of the moaR gene carried by plasmid pAT11 on the synthesis of arylsulfatase and of tyramine oxidase was examined (Table 2). When plasmid pAT11 was present in mutant strains K801 (moaR), K611 (atsT, tynA), and MKN204 (atsT, tynA), the repression of arylsulfatase by sulfate was relieved by the addition of tyramine. A restriction map of the cloned chromosomal fragment in pAT11 was constructed. The gene that complemented the moaR mutation was located within a 1.3-kb fragment (Fig. 1).

In strain W70, the synthesis of tyramine oxidase was induced by tyramine. The mutant strains K801, K611, and MKN204 failed to synthesize tyramine oxidase even when tyramine was present. After transfer of the plasmid pAT11 into strain K801, but not in strain K611 or MKN204, the synthesis of tyramine oxidase was inducible by tyramine. Since transformants of strain K13 (tynA13) and MKN63 (tynA63) carrying pAT11 did not have any monoamine oxidase activity, we concluded that pAT11 did not contain the maoA or tynA genes and did contain moaR that seems to be allelic to atsT.

Strain K801 carrying pAT11 had rather high levels of tyramine oxidase and arylsulfatase activities, even without tyramine. This phenomenon may be the result of overproduction of the gene product from the multiple-copy plasmid pAT11. Therefore, we subcloned the 1.3-kb fragment containing moaR on plasmid pAT11 into a low-copy-number plasmid, pMW219, which has a pSC101 replicon and is present as only a few copies per host chromosome (29). The resultant plasmid, pATW4, was introduced into strain K801 and the activities of tyramine oxidase and arylsulfatase

with and without tyramine were examined. No activity of either enzyme was found in the absence of tyramine. These enzymes were induced by tyramine. These results show that the gene that complements the mutation that is responsible for failure in the derepressed synthesis of arylsulfatase by tyramine also induces tyramine oxidase.

Disruption of the moaR gene on the chromosome. The moaR gene that was inactivated by insertion of the Km^r gene, on a heat-labile replicon containing plasmid pEL3 (4), was recombinated with the chromosomal DNA of K. aerogenes W70. About 2% of the transformants were defective in the derepressed synthesis of arylsulfatase in the presence of tyramine. The resultant mutant strain (MK1) showed the same phenotype as strains K801, K611, and MKN204 (Table 2).

Induction of production of a 30-kDa protein. We recently found that a 30-kDa protein of unknown function was also induced by tyramine in the wild-type strain W70 carrying a plasmid (pMA1) that included the region downstream of the atsBA operon. Its synthesis was not induced in the moaR mutant K801 carrying this plasmid. Its synthesis was induced by tyramine in strain K801 that carried the plasmid pATM1, which has the 1.3-kb fragment for the moaR gene cloned into plasmid pMA1 (Fig. 2).

Induction of tyramine oxidase, arylsulfatase, and the 30-kDa protein was induced in response to dopamine, octopamine, and norepinephrine, in addition to tyramine. The gene on pAT11 seems to have a positive regulatory effect on monoamine-induced operons. Thus, we designated the positive regulatory gene moaR instead of atsT.

Nucleotide sequence of the positive regulatory gene. The complete nucleotide sequence of the 1.3-kb fragment containing the moaR gene was determined (Fig. 3). We found an open reading frame (ORF) capable of encoding a protein of about 26-kDa. This ORF consists of 684 bp, with a putative ATG initiation codon at position 393 and a TAA termination codon at position 1074. The putative initiation codon is 8 nucleotides from a potential ribosome-binding site (gGGAGG). In the 3'-flanking region of this ORF, we found a palindromic sequence [ΔG;-19.3 kcal (ca. -80.9 kJ) /mol] which may act as a ρ-independent terminator.

Analysis of the promoter for the moaR gene. To identify the promoter region of the moaR gene, a moaR'::lacZ transcriptional fusion was constructed with a promoter probe vector, pMC1403. A 0.58-kb EcoRI-BamHI fragment was subcloned into pMC1403 (Fig. 1). The resultant plasmid was named pMCAT1. The level of expression of the moaR promoter was studied by measuring the β-galactosidase activity of the fusion in cells that carried pMCAT1 and were grown with or without tyramine (Table 3). β-Galactosidase in strain W70 that carried pMCAT1 was induced by tyramine. This result suggests that the promoter region of moaR is located in the 0.58-kb EcoRI-BamHI fragment, and this promoter is regulated positively by tyramine. When plasmid pMCAT1 was transferred into the moaR mutant strain K801, β-galactosidase was not induced by tyramine (Table 3). This result suggests that the moaR promoter may be positively autoregulated by the MoaR protein.

Homology search. The amino acid sequence deduced from the

DNA sequence of the moaR gene indicated that the gene product contains 226 amino acid residues and has a molecular weight of 26,238. We searched for homologies between the amino acid sequence of MoaR and proteins in the SWISS and PROSEQ data bases.

We found that the C-terminal region of MoaR (amino acid residues 179-198) exhibits sequence homology to a conserved 20-amino-acid region of DNA-binding proteins. These proteins interact with their target sites through specific hydrogen bonds made by amino acid side chains present on a characteristic helix-turn-helix motif. The sequence in MoaR is compared with those in several other regulatory proteins in Figure 4. Several proteins are members of response regulators and several others like MoaR are not. As in the case of proteins MalT and GerE (24), no domain homologous to a response regulator was found in MoaR.

Expression of the product of the moaR gene. To express the gene product of moaR, the moaR gene was located under control of a T7 promoter in an expression vector pVEX11. The 1.3-kb KpnI-HindIII fragment was subcloned into pVEX11. The resultant plasmid was named pVEXKH. The product derived from the plasmid pVEXKH was produced under control of the T7 expression system. Its molecular weight was approximately 26,000, as determined by SDS-PAGE and autoradiography (Fig. 5), coinciding with the molecular weight deduced from the DNA sequence.

DISCUSSION

We isolated a mutant strain of *K. aerogenes* in which the arylsulfatase synthesis is not derepressed by tyramine. This strain is also negative for tyramine oxidase activity and the production of a 30-kDa protein that is specifically induced by high concentrations of tyramine and dopamine.

Previously, Okamura et al. isolated three *atsT* mutant strains, namely, K019, K110, and K113, which were derived from the wild-type strain W70. In these mutant strains, the arylsulfatase synthesis was not derepressed by tyramine and, moreover, no tyramine oxidase activity could be detected (21). Since Tyn^+ revertants of these mutants and the transductants obtained from the wild-type strain to an *atsT* mutant strain showed that all of the Tyn^+ cells were $atsT^+$, Murooka et al. concluded that the tyramine oxidase synthesis is essential for the derepressed synthesis of arylsulfatase by tyramine (16). Unfortunately, these *atsT* mutant strains were lost and, therefore, a similar mutant strain, designated K801, was isolated from the wild-type strain W70 in this study. We reconfirmed that this mutation was also associated with loss of the ability to synthesize tyramine oxidase (*tynA*). Furthermore, we found that the single mutation also resulted in loss of production of the 30-kDa protein. These results suggest that the mutated gene encodes a positive regulator that is common to the synthesis of arylsulfatase, tyramine oxidase, and the 30-kDa protein, which are all regulated by monoamines, such as tyramine, dopamine, octopamine, and norepinephrine. Thus, we designated the gene *moaR*

and the operons regulated by moaR as the monoamine regulon.

Since transfer of the moaR gene into strain K801 and the atsT mutant strains resulted in complementation of the moaR and atsT mutations; the derepressed synthesis of arylsulfatase, the syntheses of tyramine oxidase and the 30-kDa protein in the presence of tyramine or related monoamines, we concluded that the atsT mutation is identical to moaR, and the moaR gene encodes a common positive regulator of the monoamine regulon. This conclusion was also supported by the disruption of the moaR gene on the chromosome of the wild-type strain.

From the amino acid sequence deduced from the DNA sequence of moaR, we found homology between the C-terminal domain of MoaR and the conserved sequences in the C-terminal regions of members of the UhpA subclass (FixJ, ComA, NarL, etc.) of response regulators with two-component systems (24). The C-terminal domains of members of each subclass have subclass-specific helix-turn-helix motifs for binding to DNA (24). However, in the MoaR sequence, no conserved sequence for phosphotransfer in the N-terminal region was found. It seems likely that the MoaR protein regulates the operons by binding to the regulatory region of the monoamine regulon. The finding of the moaR gene that was autogenously regulated may provide more interesting role of the monoamine regulon in living cells.

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TABLE 1. List of bacterial strains and their characteristics

Strain	Relevant genotype	Source or reference
K. aerogenes		
W70	wild-type	MacPhee et al. (12)
K801	*moaR*	This paper
K13	*tynA13*	Adachi et al. (2)
K611	*tynA13*, *atsT11*	Murooka et al. (16)
MKN63	*tynA63*	Murooka et al. (18)
MKN204	*tynA63*, *atsT204*	Mutagenesis of MKN63
E. coli		
JM109	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, λ^-, Δ(*lac-proAB*), *relA1*, [F', *proAB*$^+$, *lacI*^{q}*Z* *M15*, *traD36*]	Yanisch-Perron et al. (30)
BL21(DE3)	*hsdS*, *gal* (*lcI857*, *ind1*, *Sam7*, *nin5*, *lacUV5-T7gene1*)	Yamada and Nakazawa (8, 25)

TABLE 2. Levels of monoamine (tyramine) oxidase and arylsulfatase induced by tyramine[a]

Strain (genotype)	Plasmid	Sulfur source (3 mM)	Tyramine (3 mM)	Enzymatic activity[b] (mU/mg of protein)	
				Monoamine Oxidase[c]	Arylsulfatase
W70	-	Methionine	-		541
(wild-type)	-	Na_2SO_4	-	<0.01	2
	-	Na_2SO_4	+	7.24	315
	pAT11	Na_2SO_4	-	2.99	312
	pAT11	Na_2SO_4	+	9.54	428
K13	-	Na_2SO_4	-	<0.01	5
(*tynA13*)	-	Na_2SO_4	+	<0.01	237
	pAT11	Na_2SO_4	-	<0.01	27
	pAT11	Na_2SO_4	+	<0.01	339
MKN63	-	Na_2SO_4	-	<0.01	2
(*tynA63*)	-	Na_2SO_4	+	<0.01	143
	pAT11	Na_2SO_4	-	<0.01	73
	pAT11	Na_2SO_4	+	<0.01	248
K801	-	Methionine	-		843
(*moaR*)	-	Na_2SO_4	-	<0.01	1
	-	Na_2SO_4	+	<0.01	1
	pAT11	Na_2SO_4	-	5.44	442
	pAT11	Na_2SO_4	+	6.80	384
	pATW4	Na_2SO_4	-	<0.01	4
	pATW4	Na_2SO_4	+	4.28	268
K611	-	Methionine	-		575
(*atsT11*,	-	Na_2SO_4	-	<0.01	2
tynA13)	-	Na_2SO_4	+	<0.01	2
	pAT11	Na_2SO_4	-	<0.01	117
	pAT11	Na_2SO_4	+	<0.01	153
MKN204	-	Methionine	-		313
(*atsT204*,	-	Na_2SO_4	-	<0.01	<1
tynA63)	-	Na_2SO_4	+	<0.01	1
	pAT11	Na_2SO_4	-	<0.01	171
	pAT11	Na_2SO_4	+	<0.01	183
MK1	-	Methionine	-		201
(*moaR::kan*)	-	Na_2SO_4	-	<0.01	2
	-	Na_2SO_4	+	<0.01	<1

[a]The cells were grown in K medium with the sulfur source indicated, in the presence (+) or absence (-) of tyramine (3 mM). The cells were harvested after approximately three doublings of cell number.

[b]Values are averages of results from three independent experiments.

[c]Radiometric procedure with [^{3}H]-tyramine was used. This assay allows activities of both tyramine oxidase and monoamine oxidase to be detected (20).

TABLE 3. Effects of tyramine on regulation of the expression of *moaR* in *K. aerogenes* that carry plasmid pMCAT1[a].

Strain (genotype)	Tyramine (3 mM)	β-Galactosidase activity (U / mg of cells)
W70	-	498
(wild-type)	+	2157
K801	-	484
(*moaR*)	+	507

[a]Cells were grown in K medium in the presence (+) or absence (-) of tyramine; cells were harvested after three doublings of cell number.

LEGEND FOR FIGURES

Fig. 1. Restriction endonuclease map of pAT11 and construction of pMCAT1. The arrows show gene orientations. The construction of pMCAT1 is described in the text.

Fig. 2. Induction of 30-kDa protein by tyramine. lane1, 2: W70 (pMA1), lane3: K801 (pMA1), lane4: K801 (pATM1). For lane1, cells were grown in K medium in the absence of tyramine and for lanes 2 to 4, cells were grown in K medium in the presence of 20 mM tyramine.

Fig. 3. Nucleotide sequence of the 1.3-kb fragment and the predicted amino acid sequence of moaR. The presumptive ribosome-binding site (SD) is indicated. The horizontal arrows show inverted-repeat sequences. The DDBJ/GenBank/EMBL accession number is D15072.

Fig. 4. Comparison of the amino acid sequence of the highly conserved C-terminal region of the product of the moaR gene with the DNA-binding regions of regulators containing some response regulators. Identical amino acids are shaded.

Fig. 5. Expression of plasmid-encoded moaR. The ^{35}S-labeled proteins synthesized by T7 expression system from BL21 (pVEX11) (control; lane1) or strain BL21 (pVEXKH) (lane 2) were separated by SDS-polyacrylamide gel electrophoresis. The arrow head shows the MoaR protein.

Fig. 1.

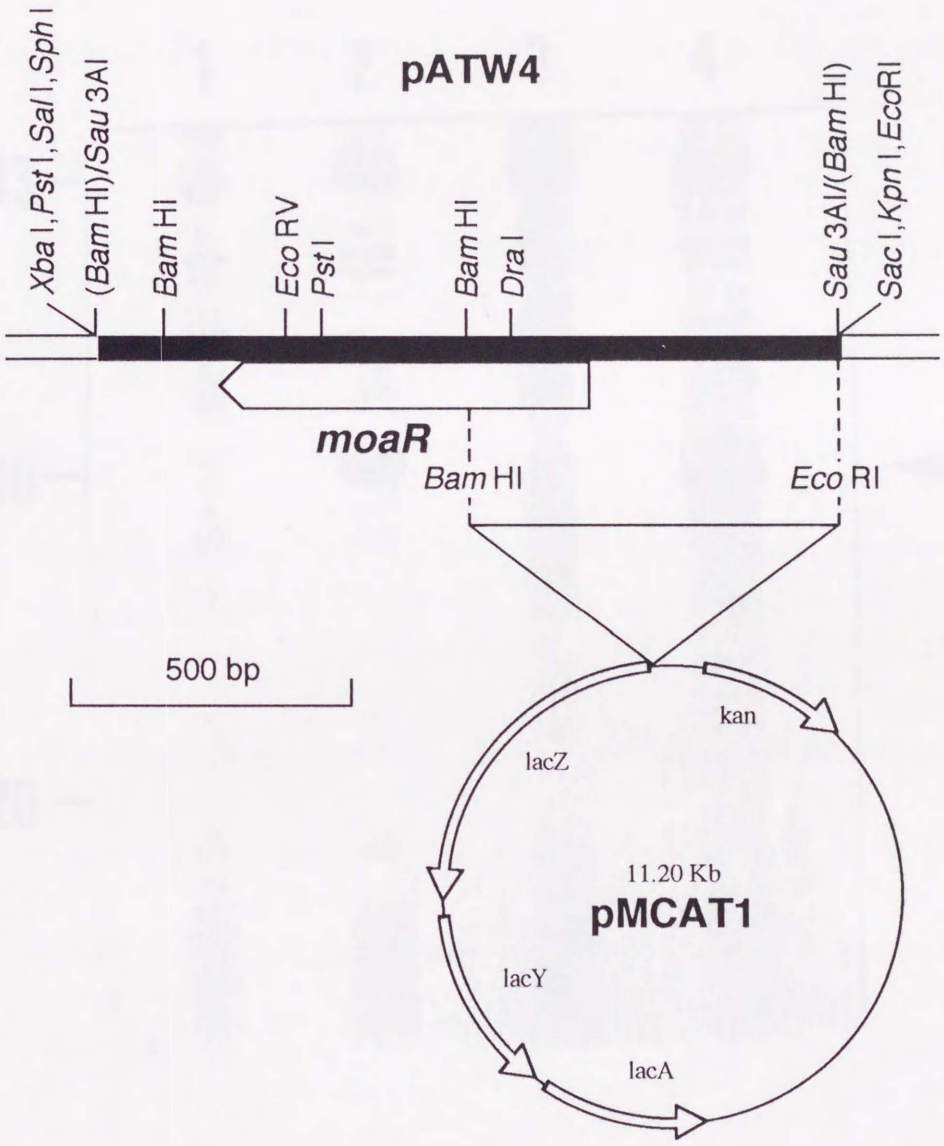
pATW4
Xba I, Pst I, Sal I, Sph I
(Bam HI)/Sau 3AI
Bam HI
Eco RV
Pst I
Bam HI
Dra I
Sau 3AI/(Bam HI)
Sac I, Kpn I, EcoRI
moaR
Bam HI
Eco RI
500 bp
kan
lacZ
11.20 Kb
pMCAT1
lacY
lacA

Fig. 2.

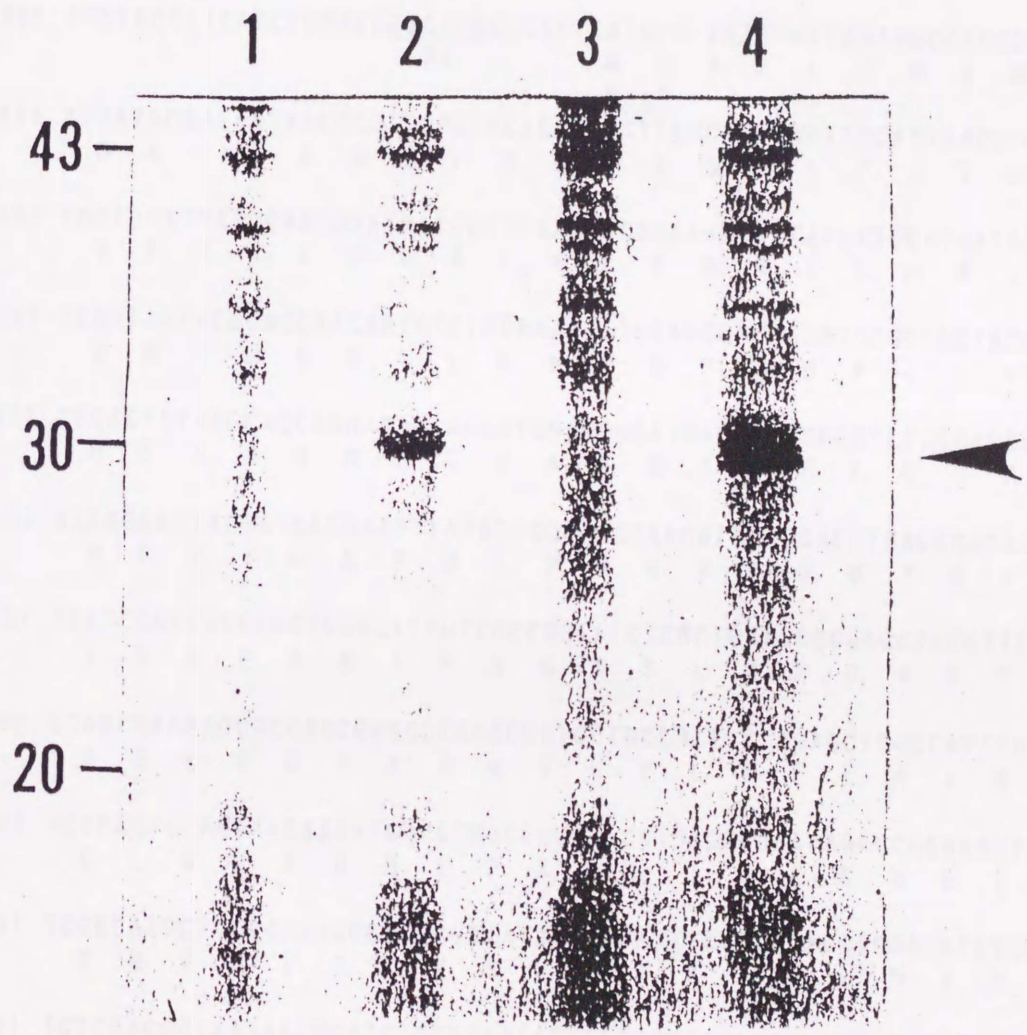

Fig. 3

```
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  61 AAATGCATCCTGTTAAGGTATAAGTGAAAAAAAACAAAAATCCATCGTTCTTTAGGATGA
 121 GAATCACGCGTCGGGAATAGGGTTTTTTTATCACCAGGGGCAGGTACGCCGGGTGTTAAT
 181 CTTATTTGTTTATGTTTTAACGCCGCTATCGTAAAGCATGGCTGGGGTGTTTTTTAAATG
 241 CTTTATATAACAATGTATTGCTGAAAATTTTGTAACGGCTATCACATTGTTAATATGTTA
 301 ATGATTGTGTTACTTAAATGTGAAATGTATGCTTCAATTATCAGTAGTCATCCATGTGTC

 361 ATGTACCATCATCTCGACGGGGAGGCCCTATCATGTCTGCTTTGTTAAAAGCCAGCCGTA
                         SD          M  S  A  L  L  K  A  S  R  N
                                     MoaR→
 421 ATGATGCGATAATAGCCCGTTGTTTACAGACTATTTCCCAGTTAATTCCACTAACTTCAG
       D  A  I  I  A  R  C  L  Q  T  I  S  Q  L  I  P  L  T  S  A

 481 CGGTGTTTTATCGAGTAAATAATCGTTTAAAACCCGAAAACTATATATTGCATAATATTT
       V  F  Y  R  V  N  N  R  L  K  P  E  N  Y  I  L  H  N  I  S

 541 CCGATAATACGCACCAACAATATCTGGAAAACTTCCAGCCGCTGGATCCGCTGCTACCGT
       D  N  T  H  Q  Q  Y  L  E  N  F  Q  P  L  D  P  L  L  P  S

 601 CGCACTTTAGCCACCAGAACACCACCGTGGCGGCGATGACGCCGCGGCTCTGCGACCGCA
       H  F  S  H  Q  N  T  T  V  A  A  M  T  P  R  L  C  D  R  N

 661 ACCGGCATTACTATCATGAATTTATGTTGCCGAATAACGTGCGCGACATGACCGAGATCT
       R  H  Y  Y  H  E  F  M  L  P  N  N  V  R  D  M  T  E  I  F

 721 TTATCCGCCGCGAGCGGCGGATCGTCGCCGGTATCTCGCTGATGCGCGACGTGCCTTTCT
       I  R  R  E  R  R  I  V  A  G  I  S  L  M  R  D  V  P  F  S

 781 CCAGCGAAGAGCGCCAGCGGGCCCAGGCGGTGCTGCCGCTGGTAGAGCTGGCCATTCGCG
       S  E  E  R  Q  R  A  Q  A  V  L  P  L  V  E  L  A  I  R  D

 841 ACTGCCTGCAGGAAGAAGATGATCTGCCCGCCATCCTGACGGCGAAAGAGCGGGAAATCG
       C  L  Q  E  E  D  D  L  P  A  I  L  T  A  K  E  R  E  I  V

 901 TCGGCATGGTGCGCGAAGGCGCCAGCAATAAGCTGATTGCCCGCCAGCTGGATATCTCGC
       G  M  V  R  E  G  A  S  N  K  L  I  A  R  Q  L  D  I  S  L

 961 TCTCGACGGTAAAAACGCACCTGCGCAATATTTTCGCCAAGACCGAAGTGGTCAATCGTA
       S  T  V  K  T  H  L  R  N  I  F  A  K  T  E  V  V  N  R  T

1021 CCGAACTGGTTTCCCGAACCTGGATGCCGGCCGCTCAGCGTACGCTGCATCTGTAATCTG
       E  L  V  S  R  T  W  M  P  A  A  Q  R  T  L  H  L  *

1081 ACTTTCGCATCCCGCGCGCGCCGTGGCAGCACGGCGTTTTCCTGATGCCTGTGGGTATCA

1141 CTCCCTGGAGGATCCCCCGGGGGGAGGCTTTTTTGACCGGAGCACGCCCTACGATGTCGA

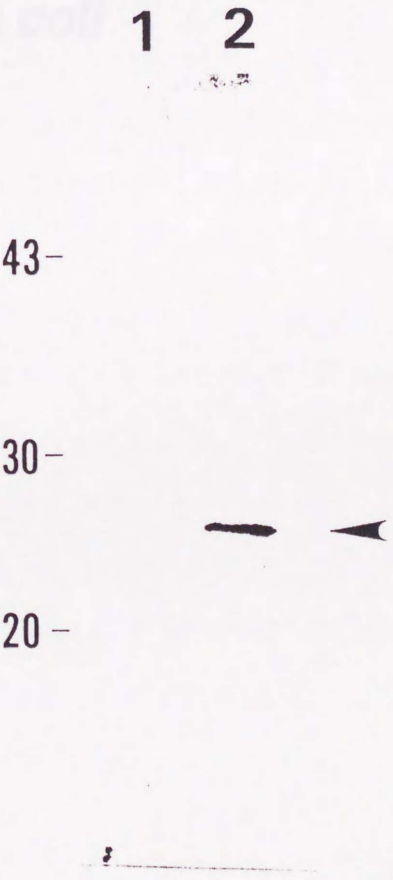
1201 GTACACCTCTCACTGATAATGCGCTTTCCCGGCCCGCCGGGCTGGTGGTGTCCCTGCGTT
1261 TGCTGGCGGCGATC
```

helix-turn-helix

MoaR	(Klebsiella)	S NKLIARQLDISLSTVKTHLR NIFAK
NarL	(E. coli)	P NKMIARRLDITESTVKVHVK HMLKK
MalT	(E. coli)	S NEQIAGELEVAATTIKTHIR NLYQK
UhpA	(E. coli)	A VKEIAAELGLSPKTVHVHRA NLMEK
FimZ	(E. coli)	S NKEIADKLLLSNKTVSAHKS NIYGK
FimZ	(Salmonella)	S NKEIAEQLLLSNKTISAHKA NIFSK
GerE	(Bacillus)	T TKEIASELFISEKTVRNHIS NAMQK
Com1	(Bacillus)	T NQEIADALHLSKRSIEYSLT SIFNK
DegU	(Bacillus)	S NRGIGESLFISEKTVKNHVS NILQK
FixJ	(Bradyrhizobium)	S NKLIAREYDISPRTIEVYRA NVMTK
FixJ	(Rhizobium)	P NKSIAYDLDISPRTVEVHRA NVMAK
AlkS	(Pseudomonas)	S NKQIATNMHVTEDAIKWHMR KIFAT

Fig. 4

Fig. 5



第三章

Nucleotide Sequence of the Gene for Monoamine Oxidase (*maoA*) from *Escherichia coli*

ABSTRACT

We found that the structural gene for monoamine oxidase was located at 30.9 min on the Escherichia coli chromosome. Deletion analysis showed that two amine oxidase genes are located in this region. A nucleotide sequence of one of the two genes was determined. The peptide sequence of the first 40 amino acids from the N terminus of monoamine oxidase purified from E. coli agrees with that deduced from the nucleotide sequence of the gene. The leader peptide extends over 30 amino acids. The nucleotide sequence of the gene and amino acid sequence of the predicted mature enzyme (M.W. 81,295) were highly homologous to those of the $maoA_K$ gene and monoamine oxidase from Klebsiella aerogenes. From these results and analysis of the enzyme activity, we concluded that the gene encodes for monoamine oxidase ($maoA_E$). The tyrosyl residue which may convert to topa quinone in the E. coli enzyme was located by comparison with amino acid sequences at the cofactor sites in other copper/topa quinone-containing amine oxidases.

Previously, we cloned the structural gene for monoamine oxidase (*maoA*$_K$) from *Klebsiella aerogenes* (1). The *maoA*$_K$ gene is part of the *mao* operon that also includes the *maoC* gene which has an unknown function and inducibly expressed by tyramine and the related monoamine compounds (2). The *mao* operon is controlled by a positive regulator *moaR* gene in *K. aerogenes* (3). We found a region of the *Escherichia coli* chromosome that was highly homologous to the *Klebsiella maoA* gene. The potential *maoA* gene is located at 30.9 min on the *E. coli* chromosome (2). We subcloned an 18.6-kb *Bam*HI-*Bam*HI fragment from *E. coli* clone 5F1 (4) into pKI212 (5), and the resultant plasmid pMOEB1 was transferred to *K. aerogenes* MKN63 that is deficient in monoamine oxidase (*maoA*) (6). The transformants carrying pMOEB1 complemented the *maoA* mutation of *K. aerogenes* (2).

Several deletion plasmids from pMOEB1 were constructed by subcloning of fragments or digestion with appropriate restriction enzymes (Fig. 1). The properties of these plasmids were analyzed by the productivity of amine oxidase in *E. coli* W3110 and by complementation tests with *K. aerogenes* MKN63 (*maoA*). *Klebsiella* cells were grown aerobically at 28° C in K-xylose medium (7) and *E. coli* cells were grown in M9-succinate including thiamine. Tyramine (3 mM) was used as an inducer of monoamine oxidase (8). Amine oxidase activity was assayed by a radiometric procedure with [^{3}H]tyramine (6) and by a colorimetric assay coupled with peroxidase and either *o*-dianisidine (9) or *o*-phenylenediamine (1). Deletion analysis suggests that two regions essential to produce amine oxidases are located within a 3.6-kb *Pvu*II-*Pvu*II

fragment (pMOSP3) and a 11.1-kb PvuII-PvuII fragment (pMOLP4), respectively (Fig. 1). By using the clone of the K. aerogenes maoCA genes, the analogous location of the potential $maoA_E$ gene in E. coli was mapped. The $maoA_K$ probe (2.6-kb SmaI-SmaI fragment) (2) hybridized strongly to the 11.1-kb PvuII-PvuII fragment but not to the 3.6-kb PvuII-PvuII fragment. The maoC probe (1.3-kb EcoRI-SmaI) (2) did not hybridize to any fragment. The cells of K. aerogenes MKN63 (tynA) carrying a plasmid pMOLP4 containing the 11.1-kb PvuII-PvuII fragment showed monoamine oxidase activity by radiometric procedure with [^{3}H]tyramine and colorimetric procedure (Fig. 1). In the cells carrying a plasmid pMOSP3 containing the 3.6-kb PvuII-PvuII fragment, however, high activity was detected only by colorimetric procedure with tyramine as the substrate and with o-phenylenediamine or o-dianisidine as a coupling indicator. This result suggests that the gene for unknown amine oxidase is located in the 3.6-kb PvuII-PvuII fragment. Thus, the gene in the 3.6-kb PvuII-PvuII fragment was tentatively named maoX.

The nucleotide sequence of the 4.9-kb PvuII-EcoRI fragment, which carries the $maoA_E$ gene, was determined (Fig. 2). Sequencing reactions were performed by the M13 dideoxy-chain termination method (10) with an Autoread T7 sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden). We found an open reading frame capable of coding for about 84.4-kDa protein. The ORF contains 2,274 bp with an ATG initiation codon at position 925 and a TGA termination codon at position 3,196. The putative initiation codon is preceded by the Shine-Dalgarno sequence GAGG (11) and a

sequence with a similarity to the -10 consensus sequence (gATAAT) but a low similarity to the -35 region (caGgCA) of promoter . In the 3'-flanking region of the ORF, we found a palindromic sequence (ΔG, -20.8 kcal [ca.-87.4 kJ]/mol) that may act as a ρ-independent terminator. This ORF was 75.0 % homologous to the DNA sequence and 83.2 % homologous to the amino acid sequence of Klebsiella monoamine oxidase gene, $maoA_K$ (Fig. 3).

To identify the product of the $maoA_E$ gene and the processing site that gives rise to the mature protein, we determined the N-terminal amino acid sequence of the purified monoamine oxidase from the periplasmic fraction of E. coli JM109 cells that carried a plasmid pMOEB1 containing the $maoA_E$ gene. The first 40 amino acids were identified as His-Gly-Gly-Glu-Ala-His-Met-Val-Pro-Met-Asp-Lys-Thr-Leu-Lys-Glu-Phe-Gly-Ala-Asp-Val-Gln-Trp-Asp-Asp-Tyr-Ala-Gln-Leu-Phe-Thr-Leu-Ile-Lys-Asp-Gly-Ala-Tyr-Val-Lys. This sequence is identical to the directly deduced amino acid sequence from position 31 to 70 of the ORF (Fig. 2). From these results and the enzyme activity in the cells carrying pMOLP4, we concluded that the found ORF is maoA, which encodes the structural gene for a monoamine oxidase. The leader peptide extends over 30 amino acids which showed typical characteristics of a signal peptide.

Previously, Cooper et al. (12) reported that amine oxidase of E. coli contained copper/topa quinone as the cofactor. However, they did not determine the amino acid sequence of the enzyme. At residue 475-477 in the amino acid sequence deduced from DNA sequence of $maoA_E$, we found the predicted cofactor site (-Asn-Tyr-Asp-) which exsists in other copper/topa quinone-containing monoamine oxidases from Klebsiella (9), Arthrobacter (13)

and Hansenula polymorpha (14) (Table 1 and Fig. 2). The tyrosyl residue is most probably converted to topa quinone in the mature enzyme as shown in other amine oxidases (12, 13, 15).

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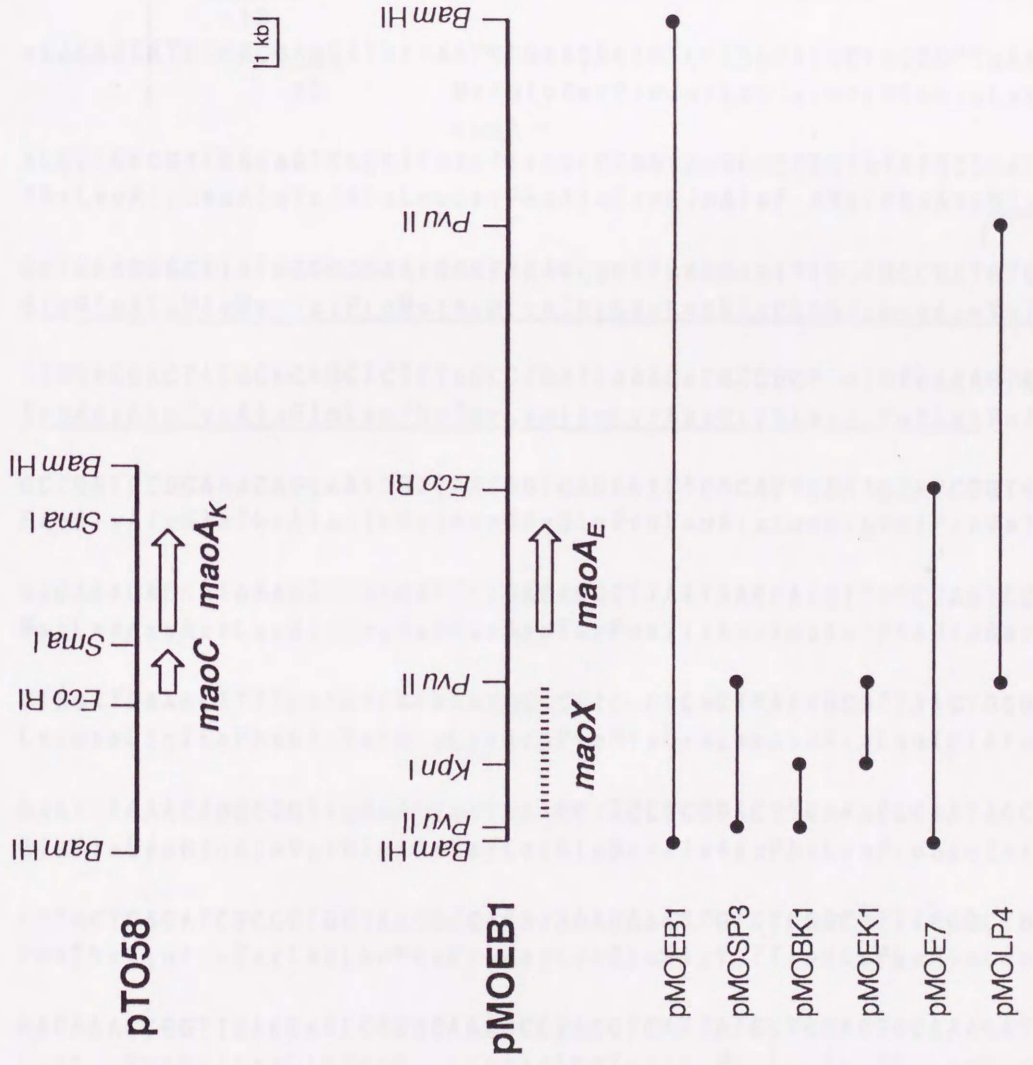
LEGEND FOR FIGURES

Fig. 1. Restriction endonuclease map and deletion analysis of pMOEB1. Deletion plasmids were transferred to *K. aerogenes* MKN63 (*tynA*) (1) and assayed for amine oxidase activities by radiometric procedure with [^{3}H]tyramine and colorimetric procedure as described in the text. Homology to $maoA_K$ was detected by Southern hybridization using the $maoA_K$ and *maoC* genes from pTO58 (2) as probes. ND, not done.

Fig. 2. Nucleotide sequence of the 3.2-kb fragment and the predicted amino acid sequence of $maoA_E$. The presumptive ribosome-binding site (SD) and possible promoter region (-10 and -35) are indicated. The horizontal arrows show inverted repeat sequence. The N-terminal amino acid sequence of the monoamine oxidase from *E. coli*, determined by automatic Edman degradation method, is shown by double line. The detail procedure of purification of the enzyme will be published elsewhere. The predicted cofactor site for copper/topa quinone is boxed. The arrow showed the processing site of the precursor enzyme. The DDBJ/GenBank/EMBL accession number is D23670.

Fig. 3. Comparison of amino acid sequences of monoamine oxidases from *E. coli* and *K. aerogenes*. Identical amino acids are indicated by the shaded boxes. The predicted cofactor sites for copper/topa quinone are shown by underline. Arrows showed processing sites.

Fig. 1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Sma I
Sma I
Eco RI
Bam HI
maoC
maoA_K
1 kb
pMOEB1
Bam HI
Pvu II
Kpn I
Pvu II
Eco RI
Pvu II
Bam HI
maoX
maoA_E
Activity of monoamine oxidase
Homology to maoA_K
pMOEB1 + +
pMOSP3 + -
pMOBK1 - ND
pMOEK1 - ND
pMOE7 + +
pMOLP4 + +

Fig. 2.

```
   1  CGATCCTACCCAGGGCCGTATCAGCCGACCGTAAAGAGCCGTTGCGTAGTGCGGAATGTG
  61  CCATGAACTTCCGTGATGATTGTATGTGGAAGGTGATGCCAGCACTGGCAGCAGGCTGTT
 121  CAATCGTGAATAAAGCTTCGGAAACCACGCCACTGACGATGTTGCGCGTGGCGGAACTGG
 181  CCAGCGAGGCTGGTATCCCTGATGGCGTTTTTAATGTCGTCACCGGGTCAGGTGCTGTAT
 241  GCGGCGCGGCCCTGACGTCACATCCTCATGTTGCGAAAATCAGTTTTACCGGTTCAACCG
 301  CGACGGGAAAAGGTATTGCCAGAACTGCTGCTGATCACTTAACGCGTGTAACGCTGGAAC
 361  TGGGCGGTAAAAACCCGGCAATTGTATTAAAAGATGCTGATCCGCAATCCCTTATTGAAG
 421  GCTTGATGACCGGAAGCTTCAATTGTATTAAAAGATGCTGATCCGCAATGGGTTATTGAA
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 541  GTGCGTCTCACCAGAAGAAAAACCAACAGGTGTGGTGGAATGGGCTGTAGAGGTATTCAA
 601  TCAGCATCAAACCCCGGTGGCGCTGTATTCAATTCTGACGCTGGTGGCCAGGCAGCACGG
 661  TGATTTTGTCGATTAATCGGTGAATGAAGGGCAACGGCGAATAGTTGCCCTTTTATTTCA
 721  CTAAGTTTTGTGACGTTGTCACATATATGCTGATGTGTACATCTATTTTCAGGGCATCCA
 781  CTGTATGAAAAGCTGGGCACACCTGCCAAACAACCTGGCAGGTGCAGGCAATCCCCTTTG
                                                    (-35)
 841  CATCAGTACTGATAATGTGAACCTGACTAAACCGCCCACAGAGCGCGGTTGCTAACAAGA
                 -10
 901  ACACAACATCTGACGAGGTTAATAATGGGAAGCCCCTCTCTGTATTCTGCCCGTAAAACA
                      SD        MetGlySerProSerLeuTyrSerAlaArgLysThr
                                maoA→
 961  ACCCTGGCGTTGGCAGTCGCCTTAAGTTTCGCCTGGCAAGCGCCGGTATTTGCCCACGGT
      ThrLeuAlaLeuAlaValAlaLeuSerPheAlaTrpGlnAlaProValPheAlaHisGly
                                                          ↑
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1081  TGGGACGACTACGCCCAGCTCTTTACCCTGATTAAAGATGGCGCGTACGTGAAAGTGAAG
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1141  CCTGGTGCGCAAACAGCAATTGTTAATGGTCAGCCTCTGGCACTGCAAGTACCGGTAGTG
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1201  ATGAAAGACAATAAAGCCTGGGTTTCTGACACCTTTATTAACGATGTTTTCCAGTCCGGG
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1261  CTGGATCAAACTTTCCAGGTAGAAAAGCGCCCTCACCCACTTAATGCGCTAACTGCGGAC
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1321  GAAATTAAACAGGCCGTTGAAATTGTTAAAGCTTCCGCGGACTTCAAACCCAATACCCGT
      GluIleLysGlnAlaValGluIleValLysAlaSerAlaAspPheLysProAsnThrArg

1381  TTTACTGAGATCTCCCTGCTACCGCCAGATAAAGAAGCTGTCTGGGCGTTTGCGCTGGAA
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1441  AACAAACCGGTTGACCAGCCGCGCAAAGCCGACGTCATTATGCTCGACGGCAAACATATC
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1501  ATCGAAGCGGTGGTGGATCTGCAAAACAACAAACTGCTCTCCTGGCAACCCATTAAAGAC
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1621  GAAGAATTTGCCGCTGCCGTGAAGAAACGCGGTATTACTGATGCCGAAAAAGTGATTACC
      GluGluPheAlaAlaAlaValLysLysArgGlyIleThrAspAlaGluLysValIleThr

1681  ACGCCGCTGACCGTAGTTATTTTCGATGGTAAAGATGGCCTGAAACAAGATGCCCGGTTG
      ThrProLeuThrValValIlePheAspGlyLysAspGlyLeuLysGlnAspAlaArgLeu

1741  CTCAAAGTCATCATCAGCTATCTTGATGTCGGTGATGGCAACTACTGGCACATCATCGAA
      LeuLysValIleIleSerTyrLeuAspValGlyAspGlyAsnTyrTrpHisIleIleGlu

1801  AACCTGGTGGCGGTCGTTGATTTAGAACAGAAAAAAATCGTTAAGATTGAAGAAGGTCCG
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      ValValProValProMetThrAlaArgProPheAspGlyArgAspArgValAlaProAla

1921  GTTAAGCCTATGCAAATCATTGAGCCTGAAGGTAAAAATTACACCATTACTGGCGATATG
      ValLysProMetGlnIleIleGluProGluGlyLysAsnTyrThrIleThrGlyAspMet

1981  ATTCACTGGCGGAACTGGGATTTTCACCTCAGCATGAACTCGCGCGTCGGGCCGATGATC
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2041  TCCACCGTGACTTATAACGACAATGGCACAAAACGCAAAGTCATGTACGAAGGTTCTCTC
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2161  GACTCTGGTGACTACGGTATGGGCACGCTAACCTCACCAATTGCTCGTGGTAAAGATGCC
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2221  CCGTCTAACGCAGTGCTCCTTAATGAAACCATCGCCGACTACACTGGCGTGCCGATGGAG
      ProSerAsnAlaValLeuLeuAsnGluThrIleAlaAspTyrThrGlyValProMetGlu

2281  ATCCCTCGGCCTATCGCGGTATTTGAACGTTATGCCGGGCCGGAGTATAAGCATCAGGAA
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2341  ATGGGCCAGCCCAACGTCAGTACCGAACGCCGGGAGTTAGTGGTGCGCTGGATCAGTACA
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      AspAlaGlyAlaThrGlyIleGluAlaValLysGlyValLysAlaLysThrMetHisAsp

2521  GAGACGGCGAAAGATGACACGCGCTACGGCACGCTTATCGATCACAATATCGTGGGTACT
      GluThrAlaLysAspAspThrArgTyrGlyThrLeuIleAspHisAsnIleValGlyThr
```

```
2581    ACACACCAACATATTTATAATTTCCGCCTCGATCTGGATGTAGATGGCGAGAATAACAGC
        ThrHisGlnHisIleTyrAsnPheArgLeuAspLeuAspValAspGlyGluAsnAsnSer

2641    CTGGTGGCGATGGACCCAGTGGTAAAACCGAATACTGCCGGTGGCCCACGCACCAGTACC
        LeuValAlaMetAspProValValLysProAsnThrAlaGlyGlyProArgThrSerThr

2701    ATGCAAGTTAATCAGTACAACATCGGCAATGAACAGGATGCCGCACAGAAATTTGATCCG
        MetGlnValAsnGlnTyrAsnIleGlyAsnGluGlnAspAlaAlaGlnLysPheAspPro

2761    GGCACGATTCGTCTGTTGAGTAACCCGAACAAAGAGAACCGCATGGGCAATCCGGTTTCC
        GlyThrIleArgLeuLeuSerAsnProAsnLysGluAsnArgMetGlyAsnProValSer

2821    TATCAAATTATTCCTTATGCAGGTGGTACTCACCCGGTAGCAAAAGGTGCCCAGTTCGCG
        TyrGlnIleIleProTyrAlaGlyGlyThrHisProValAlaLysGlyAlaGlnPheAla

2881    CCGGACGAGTGGATCTATGATCGTTTAAGCTTTATGGACAAGCAGCTCTGGGTAACGCGT
        ProAspGluTrpIleTyrAspArgLeuSerPheMetAspLysGlnLeuTrpValThrArg

2941    TATCATCCTGGCGAGCGTTTCCCGGAAGGCAAATATCCGAACCGTTCTACTCATGACACC
        TyrHisProGlyGluArgPheProGluGlyLysTyrProAsnArgSerThrHisAspThr

3001    GGTCTTGGACAATACAGTAAGGATAACGAGTCGCTGGACAACACCGACGCCGTTGTCTGG
        GlyLeuGlyGlnTyrSerLysAspAsnGluSerLeuAspAsnThrAspAlaValValTrp

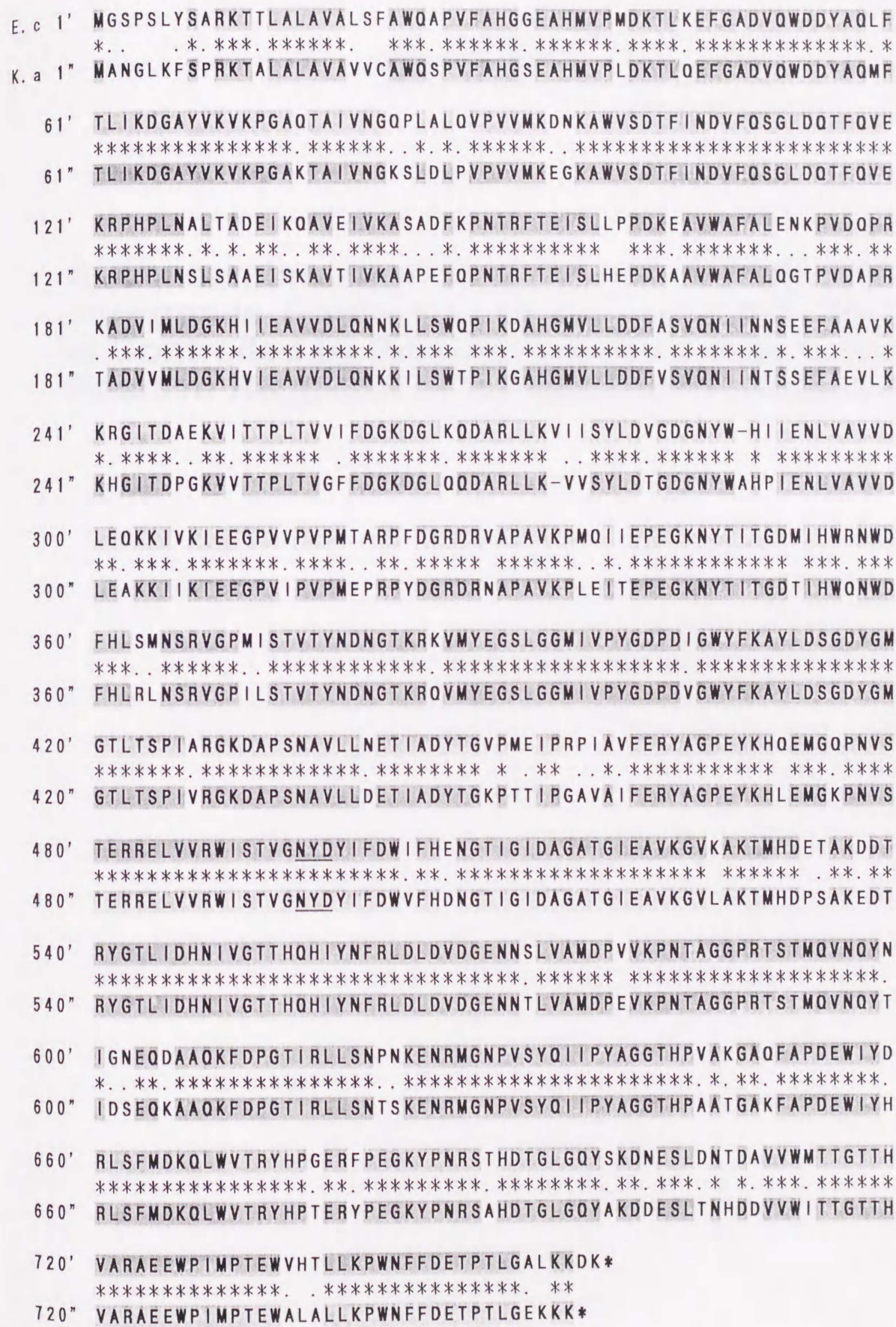
3061    ATGACCACCGGCACCACACATGTGGCCCGCGCCGAAGAGTGGCCGATTATGCCGACCGAA
        MetThrThrGlyThrThrHisValAlaArgAlaGluGluTrpProIleMetProThrGlu

3121    TGGGTACATACTCTGCTGAAACCATGGAACTTCTTTGACGAAACGCCAACGCTAGGGGCG
        TrpValHisThrLeuLeuLysProTrpAsnPhePheAspGluThrProThrLeuGlyAla

3181    CTGAAGAAAGATAAGTGATTGTTTCAGACAAAAAAACGCACCAGGTGCGTTTTTTTACTT
        LeuLysLysAspLys***

3241    ATGAGCGACCAGATTATACCGTACACACACCGACTTAGTTT
```

Fig. 3


E.c 1' MGSPSLYSARKTTLALAVALSFAWQAPVFAHGGEAHMVPMDKTLKEFGADVQWDDYAQLF
K.a 1" MANGLKFSPRKTALALAVAVVCAWQSPVFAHGSEAHMVPLDKTLQEFGADVQWDDYAQMF
61' TLIKDGAYVKVKPGAQTAIVNGQPLALQVPVVMKDNKAWVSDTFINDVFQSGLDQTFQVE
61" TLIKDGAYVKVKPGAKTAIVNGKSLDLPVPVVMKEGKAWVSDTFINDVFQSGLDQTFQVE
121' KRPHPLNALTADEIKQAVEIVKASADFKPNTRFTEISLLPPDKEAVWAFALENKPVDQPR
121" KRPHPLNSLSAAEISKAVTIVKAAPEFQPNTRFTEISLHEPDKAAVWAFALQGTPVDAPR
181' KADVIMLDGKHIIEAVVDLQNNKLLSWQPIKDAHGMVLLDDFASVQNIINNSEEFAAAVK
181" TADVVMLDGKHVIEAVVDLQNKKILSWTPIKGAHGMVLLDDFVSVQNIINTSSEFAEVLK
241' KRGITDAEKVITTPLTVVIFDGKDGLKQDARLLKVIISYLDVGDGNYW-HIIENLVAVVD
241" KHGITDPGKVVTTPLTVGFFDGKDGLQQDARLLK-VVSYLDTGDGNYWAHPIENLVAVVD
300' LEQKKIVKIEEGPVVPVPMTARPFDGRDRVAPAVKPMQIIEPEGKNYTITGDMIHWRNWD
300" LEAKKIIKIEEGPVIPVPMEPRPYDGRDRNAPAVKPLEITEPEGKNYTITGDTIHWQNWD
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360" FHLRLNSRVGPILSTVTYNDNGTKRQVMYEGSLGGMIVPYGDPDVGWYFKAYLDSGDYGM
420' GTLTSPIARGKDAPSNAVLLNETIADYTGVPMEIPRPIAVFERYAGPEYKHQEMGQPNVS
420" GTLTSPIVRGKDAPSNAVLLDETIADYTGKPTTIPGAVAIFERYAGPEYKHLEMGKPNVS
480' TERRELVVRWISTVGNYDYIFDWIFHENGTIGIDAGATGIEAVKGVKAKTMHDETAKDDT
480" TERRELVVRWISTVGNYDYIFDWVFHDNGTIGIDAGATGIEAVKGVLAKTMHDPSAKEDT
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540" RYGTLIDHNIVGTTHQHIYNFRLDLDVDGENNTLVAMDPEVKPNTAGGPRTSTMQVNQYT
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600" IDSEQKAAQKFDPGTIRLLSNTSKENRMGNPVSYQIIPYAGGTHPAATGAKFAPDEWIYH
660' RLSFMDKQLWVTRYHPGERFPEGKYPNRSTHDTGLGQYSKDNESLDNTDAVVWMTTGTTH
660" RLSFMDKQLWVTRYHPTERYPEGKYPNRSAHDTGLGQYAKDDESLTNHDDVVWITTGTTH
720' VARAEEWPIMPTEWVHTLLKPWNFFDETPTLGALKKDK*
720" VARAEEWPIMPTEWALALLKPWNFFDETPTLGEKKK*

第四章

A Monoamine-Regulated Operon in *Klebsiella aerogenes* Containing *moaE* and *moaF* That is Controlled by the Gene (*moaR*) That Is the Positive Regulator of the Monoamine Regulon

ABSTRACT

A 30-kDa protein accumulated upon induction by a high concentration of tyramine in wild-type cells of Klebsiella aerogenes that carried a plasmid (pAS123) that included the structural gene for arylsulfatase (atsA). The synthesis of the 30-kDa protein, like that of arylsulfatase and tyramine oxidase, was induced in response both to dopamine and to octopamine, as well as in response to tyramine. Deletion analysis showed that the region essential for induction of the 30-kDa protein was located within a 2.0-kb cloned segment downstream of the atsBA operon. The nucleotide sequence of the 2.0-kb fragment contained two open reading frames, moaE and moaF. The expression of a putative promoter of moaE was induced by the addition of 3 to 20 mM tyramine, and the moaF gene was transcribed from this monoamine-inducible promoter. The amino acid sequence of the first 18 amino acids from the amino-terminus of the purified 30-kDa protein agreed with that deduced from the nucleotide sequence of moaF. Using a strain with a mutant moaR gene, we found that the gene (moaR) that acts as the positive regulator of the monoamine regulon also controlled the induction of the 30-kDa protein.

INTRODUCTION

In *Klebsiella aerogenes*, arylsulfates are metabolized by an arylsulfatase (the product of the *atsA* gene), the synthesis of which is regulated by sulfur-containing compounds and aromatic monoamines. Synthesis of the enzyme is repressed by sulfate ions or cysteine (3, 4, 7) and it is induced by tyramine, dopamine, octopamine, and norepinephrine (1, 2, 7). These monoamine compounds also induce synthesis of tyramine oxidase (the product of the *tynA* gene) and monoamine oxidase (the product of the *maoA* gene) (11, 14). The *atsA* gene forms an operon with the *atsB* gene, which encodes an activator of the expression of the *atsA* gene (13). The *maoA* gene forms an operon with the *maoC* gene, the function of which is unknown (18). The expression of the *atsBA* operon, the *maoCA* operon, the *tynA* gene, and the *moaR* gene are regulated by a common positive regulator, MoaR (5). Thus, these genes belong to the monoamine regulon that is regulated by monoamine compounds (5). The MoaR protein seems to regulate the various operons by binding to the regulatory regions of the monoamine regulon. The MoaR protein is subject to autonomous control.

This report describes the specific induction of a 30-kDa protein by high concentrations of tyramine or catecholamines in *Klebsiella* cells that carry a plasmid that includes the region downstream of the *atsBA* operon. The region contains a new operon that consists of the structural gene for the 30-kDa protein and the gene for a protein homologous to insect-type alcohol dehydrogenase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. pKI212, a plasmid conferring resistance to kanamycin (Km^r) was constructed previously (13). Other recombinant plasmids were derivatives of pKI212.

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and calf intestine alkaline phosphatase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) or Toyobo Co., Ltd. (Osaka, Japan). Tyramine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). The other compounds used were standard commercial preparations.

Culture media. The rich medium used was Luria-Bertani medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl). The minimum medium used for Klebsiella strains was K medium (11), which consists of 0.5% xylose, 3 mM Na_2SO_4, 0.1% NH_4Cl, 0.01% $MgCl_2.6H_2O$, 0.001% each NaCl, $MnCl_2.4H_2O$, and $FeCl_3.6H_2O$, and 0.05 M potassium phosphate buffer (pH 7.2). The minimum medium used for E. coli was M9 medium (10).

Manipulation of DNA. Preparation of plasmid and phage DNA, restriction endonuclease digestion, ligation, and agarose electrophoresis were performed by the methods of Maniatis et al. (10). Transformation of K. aerogenes was performed by a modified version (4) of the method of Hanahan (6).

Analysis of cell extracts. Bacterial cells were grown aerobically at 28°C in K medium. The cells were concentrated and suspended in 50 mM Tris-HCl buffer (pH 7.2). Cell extracts obtained by sonication were mixed with sample buffer (pH 6.8), which contained 50 mM Tris-HCl, 2% 2-mercaptoethanol, 4% sodium

dodecyl sulfate (SDS), and 5% glycerol. After the samples had been boiled for 5 min, the proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with 0.2% Coomassie brilliant blue (CBB).

Assay of β-galactosidase activity. Bacterial cells were grown aerobically at 28°C in K medium. The activity of β-galactosidase was estimated spectrophotometrically by the method of Miller, with o-nitrophenyl-galactopyranoside (ONPG) as the substrate (15).

Analysis of DNA sequences. Sequencing reactions were performed by the M13 dideoxy chain-termination method (16) 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 nucleotide and amino acid sequences were analyzed with GENETYX programs (SDC Software Development Co., Ltd., Tokyo, Japan).

Analysis of peptide sequence. The amino-terminal sequence of the purified 30-kDa protein was determined with a protein sequencer (model 477A; Applied Biosystems, Inc., Foster City, CA).

Nucleotide sequence accession number. The DDBJ/GenBank/EMBL nucleotide sequence accession number of the fragment sequenced in this study is ------.

RESULTS

A 30-kDa protein that is induced by tyramine. In a previous study we cloned the structural gene for arylsulfatase (*atsA*) from *K. aerogenes* W70 (13). The cloned plasmid, pAS123, contained a 7.5-kb chromosomal DNA fragment that included the monoamine-regulated *atsBA* operon (13). During the purification of arylsulfatase from *K. aerogenes* W70 that carried pAS123, we found that production of a 30-kDa protein was induced by a high concentration of tyramine (20 mM). The protein was visualised after SDS-PAGE as a dense CBB-stained band. The level of the 30-kDa protein increased with increasing concentrations of tyramine from 3 mM to 20 mM. However, no dense band of a 30-kDa protein was found at concentrations of tyramine below 3 mM (Fig. 1A). Since, even in the presence of 20 mM tyramine, the 30-kDa protein was not observed in an analysis of *K. aerogenes* W70 cells without the plasmid, the 30-kDa protein seemed to be derived from plasmid pAS123.

Effects of various amines on the production of the 30-kDa protein. Various amines (each at 20 mM) were tested for their ability to induce the production of the 30-kDa protein in *K. aerogenes* W70 cells that carried pAS123 (Fig. 1B). Dopamine and norepinephrine were used at 5 mM since the growth of cells was inhibited by these amines at 20 mM. Production of the 30-kDa protein was induced in response to dopamine and octopamine, as well as in response to tyramine, as is the production of tyramine oxidase and arylsulfatase. However, these latter enzymes were not observed as dense bands on gels after SDS-PAGE. Other monoamine, diamine, and polyamine compounds had no did effect on

the production by plasmid-carrying cells of the 30-kDa protein (data not shown).

The region essential for the production of the 30-kDa protein. To identify the region essential for the production of the 30-kDa protein, a series of deletion plasmids was constructed. The deletion plasmids were introduced into K. aerogenes W70 and tested for their ability to produce the 30-kDa protein. The deletion analysis suggested that the region essential for the production of the 30-kDa protein was located within a 2.0-kb SalI-SpeI fragment, downstream from the atsBA operon (Fig. 2).

Nucleotide sequence of the region required for the production of the 30-kDa protein. The complete nucleotide sequence of the 1,967-bp SalI-SpeI fragment was determined (Fig. 3). We found two open reading frames (ORFs) capable of encoding proteins of about 26 and 29 kDa. The first ORF consists of 774 bp, with a putative ATG initiation codon at position 153 and a TAA termination codon at position 926. The putative initiation codon is preceded by a sequence that is similar to the -10 and -35 consensus sequence (gcGAgA=18 bp=TATcgT) and has a potential ribosome-binding site (AGGAGa) (17). The second ORF consists of 789 bp with an ATG initiation codon at position 952 and a TAG termination codon at position 1,740. The initiation codon, located 26 bp downstream from the termination codon of the first ORF, is preceded by the Shine-Dalgarno sequence (AGGAG). Since the two ORFs are essential for the production of the monoamine-inducible 30-kDa protein, we have tentatively designated these genes moaE and moaF, respectively.

Control of the synthesis of the 30-kDa protein by the product of the *moaR* gene. Since tyramine-inducible enzymes are regulated by the product of the *moaR* gene, we examined the synthesis of the 30-kDa protein in a strain of *K. aerogenes* with a mutant *moaR* gene. The synthesis of the 30-kDa protein was not observed in cells of the *moaR* mutant K801 that carried pMA1, which included the *moaE* and *moaF* genes. However, the synthesis of the 30-kDa protein was induced by 20 mM tyramine in cells of strain K801 that carried plasmid pATM1. This plasmid was constructed by cloning of the *moaR* gene into plasmid pMA1 (5). In *E. coli* JM109 cells that carried pMA1, the 30-kDa protein was not detected after incubation in the presence of 20 mM tyramine, whereas cells that carried pATM1 synthesized the 30-kDa protein in the presence of tyramine (Fig. 4). These results suggest that the synthesis of the 30-kDa protein is controlled by the *moaR* gene and that "wild-type" *E. coli* cells do not have the *moaR* gene or that such a gene is not functional in *E. coli*.

Identification of the gene that encodes the 30-kDa protein. We introduced plasmid pATM1 into the wild-type strain of *K. aerogenes* W70 and tested its ability to produce the 30-kDa protein. A denser band of the 30-kDa protein was observed in the case of W70(pATM1) cells than in the case of W70 cells that carried plasmid pMA1. We purified the 30-kDa protein from W70(pATM1) cells by fractionation with $(NH_4)_2SO_4$, with subsequent ion-exchange chromatography on DEAE cellulose and reverse-phase chromatography. Peak fractions of protein were subjected to SDS-PAGE to monitor the purity of the 30-kDa protein. To identify the gene for the 30-kDa protein, we determined the N-terminal sequence of

the purified 30-kDa protein. The first 18 amino acids were identified as Ala-Asp-Gly-Phe-Ala-Pro-His-Gly-Asn-Leu-Leu-Ala-Thr-Ala-Ser-Leu-Pro-Ala-. This sequence was identical to that of the directly deduced amino acid sequence from positions 14 to 31 of the second ORF (the moaF gene). Therefore, we concluded that the moaF gene encoded the 30-kDa protein whose expression was controlled by moaR.

Transcriptional analysis of the moaE and moaF genes. To identify the promoter region and to analyze the transcriptional control of the moaE and moaF genes, several lacZ transcriptional fusion plasmids were constructed using a plasmid, pMSKM, that was constructed by insertion of a kanamycin-resistance gene into a promoter probe vector, pMS437C (8). Then 0.39-kb SalI-PstI, 1.09-kb SalI-PvuII and 0.70-kb PstI-PvuII fragments were subcloned into pMSKM and the resultant plasmids were named pMSPE, pMSPEF, and pMSPF, respectively (Fig. 5). The levels of expression of the promoter were studied by measuring the β-galactosidase activity of each fusion in K. aerogenes W70 cells that carried these plasmids and had been grown with or without tyarmine. β-Galactosidase activity in strain W70 that carried pMSPE or pMSPEF was induced by tyramine. However, in strain W70 that carried pMSKM or pMSPF there was no induction of β-galactosidase activity by tyramine at any concentration tested (Table 2). These results suggest that the moaE and moaF genes are transcribed polycistronically from the moaE promoter, which is regulated by monoamine compounds. To clarify the role of the moaE gene, we deleted a 0.47-kb NaeI-StuI fragment from the moaE gene, con-

structing plasmid pDE1 (Fig. 2). The synthesis of 30-kDa protein in strain W70 that carried pDE1 was observed at the same level as that in strain W70 that carried pMA1. This result suggests that the product of the moaE gene is not essential for or is not a regulator of the expressionof moaF but that moaF is transcribed from the moaE promoter, which is activated by monoamine compounds.

Homology search. We searched for homologies between the amino acid sequences of the MoaE and MoaF proteins and proteins in the SWISS and PROSEQ databases. We found that the amino acid sequence of MoaE was 25-30% homologous to the amino acid sequences of proteins that belong to the insect-type alcohol dehydrogenase/ribitol dehydrogenase family. However, the MoaF protein was not homologous to any proteins in the databases.

DISCUSSION

In this report, we have described a new operon, the moaE-moaF operon, which belongs to the monoamine regulon that is controlled by monoamines. A 30-kDa protein is produced in increasing amounts in response to increasing concentrations of tyramine. The 30-kDa protein was induced by tyramine and also by catecholamines such as dopamine and octopamine. These compounds also induce arylsulfatase (atsA), tyramine oxidase (tynA) and monoamine oxidase (maoA). The 30-kDa protein was not observed in a moaR mutant strain of Klebsiella, K801 or in E. coli cells. However, the introduction of the moaR gene into these strains allowed induction of the 30-kDa protein. In the case of strains carrying multiple moaR genes, denser bands of the 30-kDa protein were observed than in the case of cells with the moaR gene on the K. aerogenes chromosome. From these results, we conclude that the moaE-moaF operon is an operon in the monoamine regulon that is controlled by a common positive-regulator gene, moaR.

The amino acid sequence of the amino-terminus of the 30-kDa protein purified from a hyperproducing strain with multiple moaR genes agreed with that from position 14 of the amino acid sequence deduced from the nucleotide sequence of moaF. These results suggest that the 30-kDa protein is processed between $Ala^{12}Leu^{13}$ and Ala^{14} in Klebsiella aerogenes and is secreted into the periplasm. Although accumulation of large amounts of the protein in the periplasm occurred upon induction by tyramine, the function of the 30-kDa protein remains unknown. No amine oxidase or sulfatase activity and no homology of the amino acid sequence of the 30-kDa protein to those of other known proteins were

found.

The results of the lacZ fusion analysis and the moaE deletion analysis suggest that the moaE and moaF genes are transcribed polycistronically from the moaE promoter, which is activated by the addition of monoamine compounds. The amino acid sequence of MoaE is homologous to those of proteins that belong to the insect-type alcohol dehydrogenase/ribitol dehydrogenase family. It is possible that the moaE gene might encode an enzyme that catalyzes the oxidoreduction of monoamine compounds or their metabolites.

As shown schematically in Figure 6, the moaR gene is induced by monoamine compounds, and the MoaR protein regulates at least five operons, namely, atsBA, maoCA, moaR, tynA, and moaEF, by binding to the regulatory region of the monoamine regulon. The discovery of the new operon moaEF regulated by the moaR gene may be an indication of a more interesting role for the monoamine regulon in living cells.

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Table 1. Bacterial strains and their characteristics

Strain	Relevant genotype	Source or reference
K. aerogenes		
W70	Wildtype	9
K801	*moaR*	5
MKN63	*tynA63*	12
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44* λ^- Δ(*lac-proAB*) *relA1* (F' *proAB*$^+$ *lacI*^{q}*Z M15 traD36*)	19

Table 2. Effects of tyramine on the expression of fusion of *moaE* and *moaF* with the gene for β-galactosidase in *K. aerogenes* W70[a]

Plasmid[b]	Tyramine (mM)	β-Galactosidase activity (U)[c]
pMSKM	0	230
	3	190
	20	260
pMSPE	0	150
	3	2,620
	20	8,730
pMSPEF	0	130
	3	2,570
	20	3,920
pMSPF	0	250
	3	210
	20	240

[a]Cells were grown in K medium in the presence or absence of tyramine. Cells were harvested after three doublings of cell numbers.

[b]See also Figure 5.

[c]Activities are shown in Miller units.

LEGENDS FOR FIGURES

Fig. 1 Induction of the 30-kDa protein (arrowhead) by tyramine. **(A)** Tyramine was added as an inducer as follows: lane 1, 0 mM; 2, 1 mM; 3, 3 mM; 4, 5 mM; 5, 10 mM; 6, 20 mM. **(B)** Amines were added to the medium as follows: lane 1, tyramine (20 mM); 2, dopamine (5 mM); 3, octopamine (20 mM); 4, norepinephrine (5 mM). Molecular masses are indicated in kilodaltons on the left.

Fig. 2. Restriction endonuclease map of pAS123 and derivative plasmids. The arrows show the orientation of genes. Deletion plasmids were used to transform K. aerogenes W70, and cell extracts were analyzed by SDS-PAGE.

Fig. 3. Nucleotide sequence of the 2.0-kb fragment and the predicted amino acid sequences deduced from the moaE and moaF genes. The presumptive ribosome-binding site (SD) and possible promoter regions (-10 and -35) are indicated. The arrows show the direction of moaE and moaF. The amino-terminal amino acid sequence of the purified 30-kDa protein, determined by automatic Edman degradation, is shown by double underlining. The DDBJ/GenBank/EMBL accession number is ------.

Fig. 4. Induction of the 30-kDa protein (arrowhead) by tyramine in E. coli cells. Lanes 1 and 2, JM109 (pATM1); lanes 3 and 4, JM109 (pMA1). Cells were grown in M9 medium in the absence of tyramine (lanes 1 and 3) and in the presence of 20 mM tyramine (lanes 2 and 4). Molecular masses are indicated in kilodaltons on the left.

Fig. 5. Construction of lacZ-fusion plasmids, pMSPE, pMSPEF, and pMSPF. The arrows show the orientation of genes. The construction of the plasmids is described in the text.

Fig. 6 Schematic model of monoamine regulon that is regulated by MoaR. The moaR gene is expressed upon induction by monoamines, such as tyramine, octopamine, dopamine, norepinephrine, via a predicted sensor and signal transduction. The expression of moaR is regulated autonomously (5). The MoaR protein derepresses the atsB-atsA operon that contains the structural gene for arylsulfatase which is repressed by sulfur compounds via a negative regulator, FolA (4). MoaR also positively regulates the tynA, maoC-maoA, and moaE-moaF operons.

Fig. 1.

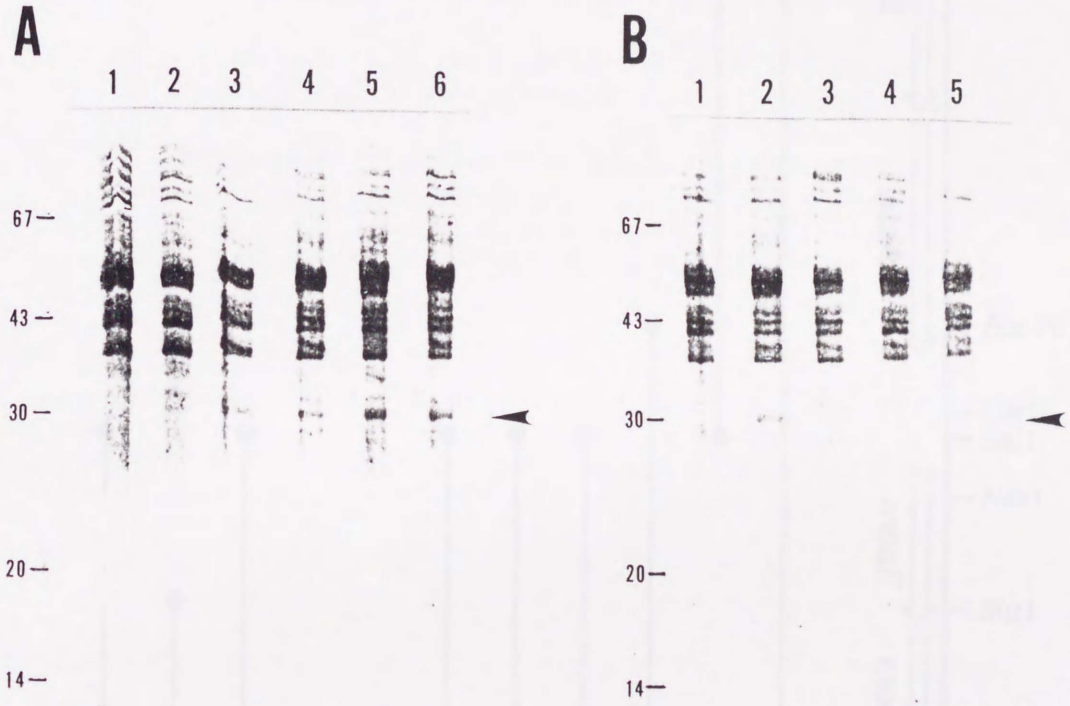

Fig. 2.

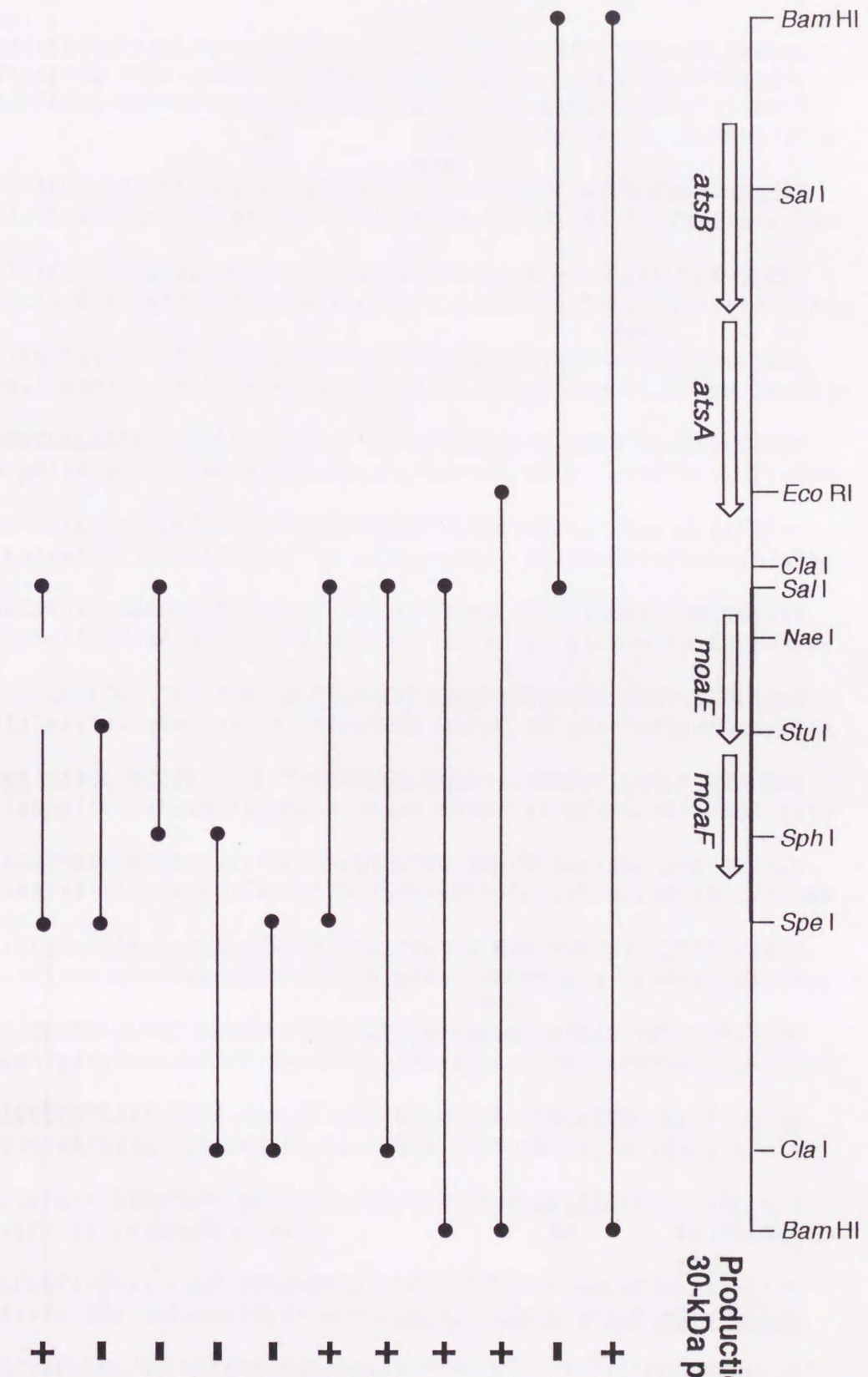
pAS123
pASS9
pASE1
pASB1
pASC1
pMA1
pASC2
pASC3
pASS7
pASS11
pDE1
+
-
-
-
-
+
+
+
+
-
+
Production of 30-kDa protein
Bam HI
Sal I
atsB
atsA
Eco RI
Cla I
Sal I
Nae I
moaE
Stu I
moaF
Sph I
Spe I
Cla I
Bam HI

Fig. 3

```
        SalI
   1 GTCGACCCCGTCACCATGAAGCCGGTCGTGGCCCGCAGGCGATCCCTGTGAGCGAGATCC
  61 ATTAACCACCACGGCTATCGTTCTTCCGGGCGATAGCCGGGACCTGACGCGCTTTTTAAG
 121 GTAATCGGAAACCCCATTACAGGAGAGTGCGGATGGCGAGAGTCGTGGTAATTACCGGCG
                          SD          MetAlaArgValValValIleThrGlyGly
                                   moaE→
 181 GTGGAACCGGAATTGGCGCTGCCTGCGCGCGGCTGATGCACCCCGCGGGCGAACGGGTGT
       GlyThrGlyIleGlyAlaAlaCysAlaArgLeuMetHisProAlaGlyGluArgValPhe

 241 TTATTACCGGACGGCGCGACGCTGTCAGGGCTGTCGCCAATGAGACCGGGGCCACGGCGC
       IleThrGlyArgArgAspAlaValArgAlaValAlaAsnGluThrGlyAlaThrAlaLeu
                                                      NaeI
 301 TGGTGGCGATGCCGCCGACGGCGAGGTGTGGCGCCAGCGGCTGCTGCCGGCGATCCTCGA
       ValAlaMetProProThrAlaArgCysGlyAlaSerGlyCysCysArgArgSerSerThr
                                PstI
 361 CCAGACCGGCGGGATTGATGTCCTCATCTGCAGCGCCGGCGGGATGGGCAACAGCCCCGC
       ArgProAlaGlyLeuMetSerSerSerAlaAlaProAlaGlyTrpAlaThrAlaProPro

 421 CGCCGAGACCAGCGACCGCCAATGAGCGCGAGGCGCTGGACGGCAATCTCACCAGCGCCT
       ProArgProAlaThrAlaAsnGluArgGluAlaLeuAspGlyAsnLeuThrSerAlaPhe

 481 TCGCCAGCGTTCGCGCCTGTCTGCCTTCGCTGATTGCCCGCCGCGGCAATGTGCTGTTTG
       AlaSerValArgAlaCysLeuProSerLeuIleAlaArgArgGlyAsnValLeuPheVal

 541 TCGCCTCCATCGCCTCTCTGGCCGCCGGGCCGCAGGCCTGCGGCTACGTCACCGCCAAAC
       AlaSerIleAlaSerLeuAlaAlaGlyProGlnAlaCysGlyTyrValThrAlaLysHis

 601 ACGCTTTAATCGGCCTGATGCGCTCCGTCGCCCGCGATTATGGCCCCCAGGGGGTACGCG
       AlaLeuIleGlyLeuMetArgSerValAlaArgAspTyrGlyProGlnGlyValArgAla

 661 CTAACGCTATCTGTCCCGGCTGGGTCACGACGCCGATGGCGGATGAAGAGATGCACCCGC
       AsnAlaIleCysProGlyTrpValThrThrProMetAlaAspGluGluMetHisProLeu

 721 TGATGCAGGCGGAAGGGCTATCGCTGACTGAGGCTTATCAGCGGGTGTGCCGCGACGTTC
       MetGlnAlaGluGlyLeuSerLeuThrGluAlaTyrGlnArgValCysArgAspValPro
                                         StuI
 781 CGCTACGCCGCCCCGCCAGCCCCGAGGAGATAGCCCAGGCCTGTCAGTTTCTCTGCTCTC
       LeuArgArgProAlaSerProGluGluIleAlaGlnAlaCysGlnPheLeuCysSerPro

 841 CGCAGGCCGCCATCATCAGCGGCGCTACGCTGGTCGCCGACGGCGGGGCCAGTATCGTCG
       GlnAlaAlaIleIleSerGlyAlaThrLeuValAlaAspGlyGlyAlaSerIleValAsp

 901 ATGTTCCCACTCTGGCGTTTGCCTAACGCCCTACTCTGTTCAGGAGCCCCTATGACTTCA
       ValProThrLeuAlaPheAla***                SD       MetThrSer
                                                     moaF→
 961 GAAGCCGTATTTATCCAGGTCGGCGCGCTGGCCGATGGTTTTGCGCCGCACGGTAACCTG
     GluAlaValPheIleGlnValGlyAlaLeuAlaAspGlyPheAlaProHisGlyAsnLeu

1021 CTGGCTACCGCCAGCCTGCCCGCCGGCGAAAACTTTACCTTTTATGTCGCCGGGAGCGAG
     LeuAlaThrAlaSerLeuProAlaGlyGluAsnPheThrPheTyrValAlaGlySerGlu
```

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          PvuII
1081 CCGCAGCAGCTGGTTATCGAAGATGAGCAGACGCTAAGCTGGAACGGCAAGCGCGCCCCC
     ProGlnGlnLeuValIleGluAspGluGlnThrLeuSerTrpAsnGlyLysArgAlaPro

1141 TGGCGAGCGACCGCCCTGCGCCCGGACATTCTGTTTATCGACTTCCTTGACCCGGAGCGG
     TrpArgAlaThrAlaLeuArgProAspIleLeuPheIleAspPheLeuAspProGluArg

1201 GATAACGCCAGCATTAGCGCGGTATGCAACCTGACGCAGCGCAATGCCACGCTGGTATAC
     AspAsnAlaSerIleSerAlaValCysAsnLeuThrGlnArgAsnAlaThrLeuValTyr

1261 GGCCAGCTGCCGGACGAAGCGCCGCGCGCTGGACGCCTTCAGCCGGGTAGAACAAGGGTT
     GlyGlnLeuProAspGluAlaProArgAlaGlyArgLeuGlnProGlyArgThrArgVal

1321 GCGCTGACCGCGGTTGAGGTCCGTTTCGTCTTCGCCCGCCTGGATGCCCAGCCGGGGCCG
     AlaLeuThrAlaValGluValArgPheValPheAlaArgLeuAspAlaGlnProGlyPro
                                        SphI
1381 CTGCCGGGCTTTACCGATGCGCTCATTGGCATGCGCAATCAGTACACCTACAGCCCGACC
     LeuProGlyPheThrAspAlaLeuIleGlyMetArgAsnGlnTyrThrTyrSerProThr

1441 GAGCGCTATGAGCACATCTATCTCAACGACAATTTTTACGCCTGGCAGTGTCTGGACGGC
     GluArgTyrGluHisIleTyrLeuAsnAspAsnPheTyrAlaTrpGlnCysLeuAspGly

1501 GTGGAAAAGGGGCTGGCGGATGTCGATCGCTGCCACTATGTGCAGGTGGCTGAGGATCTC
     ValGluLysGlyLeuAlaAspValAspArgCysHisTyrValGlnValAlaGluAspLeu

1561 TATCTGTTCGTCTGGCGGGAGAAAATCATTCCCACGCTGGGGGTGATCCTCATCGATCTG
     TyrLeuPheValTrpArgGluLysIleIleProThrLeuGlyValIleLeuIleAspLeu

1621 CAGCAGATGCGCACTGACGGCAAGATCATGGGCTATCAGGGCAGCGATTTCGGCGCCCTC
     GlnGlnMetArgThrAspGlyLysIleMetGlyTyrGlnGlySerAspPheGlyAlaLeu

1681 AGCAATTTTCCGGTCGGCGCCACGGCGAAGATCCTCAACGTCACCCGCCATCAGGAATAG
     SerAsnPheProValGlyAlaThrAlaLysIleLeuAsnValThrArgHisGlnGlu***

1741 TAGGGCGCCGCCGGGCACGCTTTCAGGCACCGCACCCGTCATTTCGCCGGGTGGCGGCTG
1801 CGCCTTACCCGGCCTACCGGGGCCTCTCACAGGCTCACACCTCGCAGGCCCGGTAAGCGC
1861 AGCGCCACCGGGCGATGCTATCAGGCACAGAGCCGCTTTATTGCCGGGTGCGTGGCGCCT
                                                  SpeI
1921 TACCCGGCTACGGTCCGTAGCAGCCAGCCGTTAACTTACCCACTAGT
```

Fig. 4

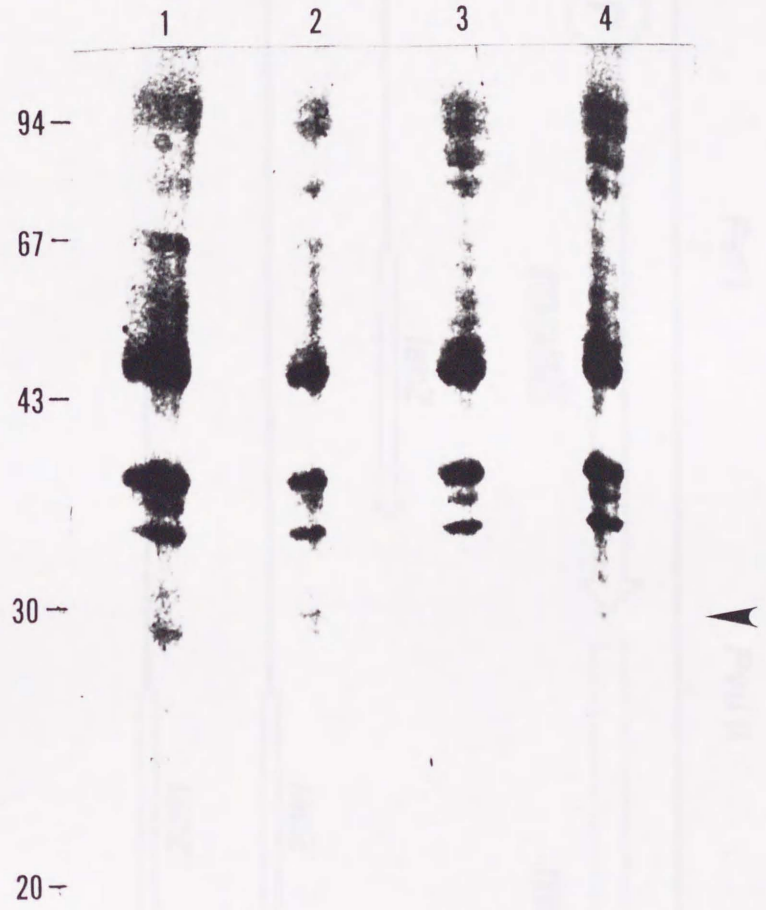
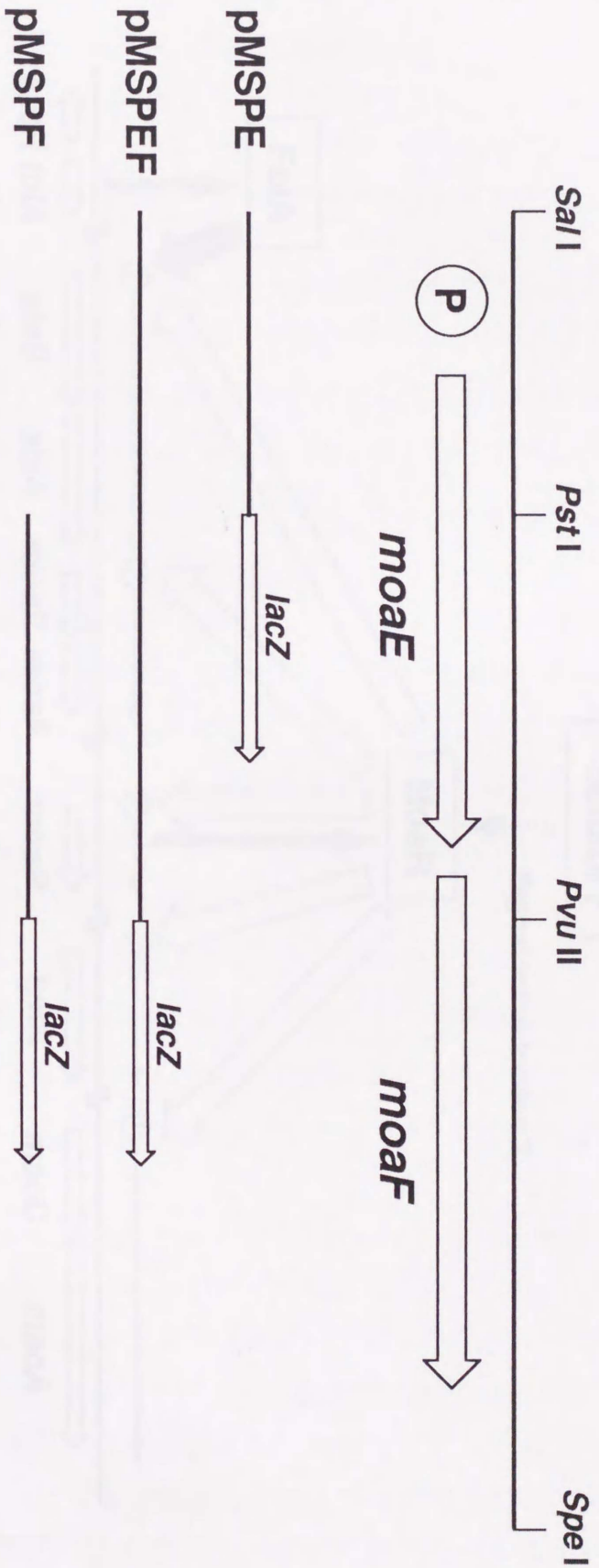
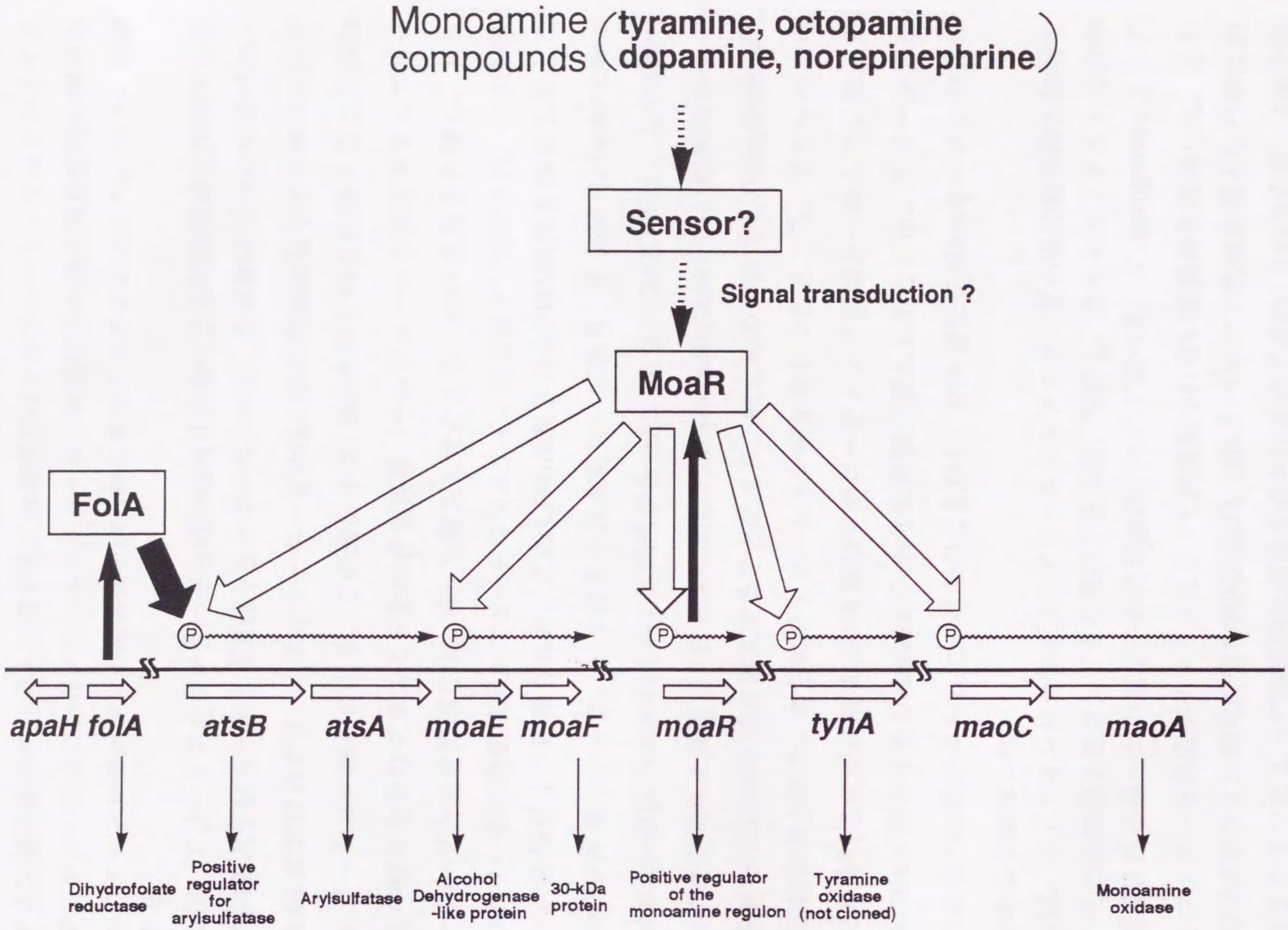


Fig. 5.


Sal I
P
Pst I
moaE
Pvu II
moaF
Spe I
pMSPE
lacZ
pMSPEF
lacZ
pMSPF
lacZ



The Monoamine Regulon

Fig. 6

腸内細菌*Klebsiella aerogenes*においてアリールスルファターゼ合成は硫黄化合物によって抑制され、モノアミン化合物によって脱抑制される。さらにこのモノアミン化合物はモノアミン酸化酵素の合成も誘導する。本研究ではサルフェートレギュロンとモノアミンレギュロンの交点であるアリールスルファターゼ遺伝子の発現調節機構の解明を中心に、モノアミンレギュロンの全体像を明らかにすることを目的とした。以下、本論文を要約する。

第一章では、アリールスルファターゼ合成の硫黄化合物による抑制機構を解明するために、その調節因子をコードする遺伝子をクローニングし、その解析について述べた。*K. aerogenes*におけるアリールスルファターゼ合成の負の調節遺伝子をクローニングした。ディリーション解析により、この遺伝子は1.6kbの断片中に位置することがわかった。この断片を含むプラスミドを*atsR*変異株（サルフェートの存在下でもアリールスルファターゼ合成を抑制しない株）に導入したところ、*atsR*変異を相補し、アリールスルファターゼの合成はサルフェートやシステインの存在下で抑制され、この抑制はチラミンの添加により解除された。この1.6kbの断片の全DNA配列を決定したところ、二つのORFが見つかった。一つはそのN末を欠いており、*E. coli*のdiadenosine tetraphosphatase遺伝子(*apsH*)と高い相同性があった。もう一つのORFは、この*apaH*-likeな遺伝子と逆向きに位置し、*E. coli*のdihydrofolate reductaseと高い相同性があった。この領域を*Klebsiella*菌に導入することにより、野生株の30倍以上のdihydrofolate reductase活性が得られた。さらにディリーション解析を行ったところ、アリールスルファターゼの抑制に必要な領域は*folA*遺伝子のコーディング領域と一致していた。また、*E. coli*の*folA*遺伝子を*Klebsiella*の*atsR*変異株に導入したところ、アリールスルファターゼ合成の抑制がみられた。以上のことから、*folA*遺伝子が*ats*オペロンの負の調節因子であると結論した。

第二章では、モノアミン化合物によるアリールスルファターゼの脱抑制機構を解明する目的で、その調節因子をコードする遺伝子を単離、解析し、さらにモノアミン化合物によって誘導される幾つかの遺伝子の発現調節について述べた。*K. aerogenes*よりアリールスルファターゼ合成がチラミンによって脱抑制されない変異株を取得した。この変異株(K801)はチラミン酸化酵素や30kDa蛋白（*ats*オペロン下流域を導入した株において高濃度チラミンによって特異的に誘導合成される分子量約3万の蛋白質；4章で詳述）のチラミンによる誘導も行われなくなった。そこでこの株を用いてアリールスルファターゼの活性化を調節する遺伝子(*moaR*)をクローニングし、そのDNA塩基配列を決定した。クローニングした遺伝子をK801株に導入したところ、モノアミン化合物に

よるアリールスルファターゼの脱抑制、及びチラミン酸化酵素、30kDa蛋白の誘導合成が回復した。また、塩基配列よりMoaR蛋白のC末領域には二成分系調節蛋白のものと相同性を示すhelix-turn-helixモチーフが見つかった。さらに*moaR-lacZ* fusionにより、MoaR蛋白は*moaR* 遺伝子自身の発現を正にautoregulationしていることがわかった。以上のことから、MoaR蛋白は*ats* オペロン、*tyn* オペロンを含む幾つかのモノアミンレギュロン遺伝子を正に調節していることがわかった。

第三章では、*E. coli* のモノアミン酸化酵素遺伝子の単離、解析について述べた。モノアミン酸化酵素の構造遺伝子が大腸菌ゲノムの30.9分付近に位置することを発見した。ディリーション解析の結果、この領域には二つのモノアミン酸化酵素が存在することがわかった。このうちの一遺伝子のDNA塩基配列を決定した。*E. coli* から精製したモノアミン酸化酵素のN末の40アミノ酸のペプチド配列は、この遺伝子のDNA塩基配列から予想されるものと一致していた。また、シグナルペプチドと思われる配列が30アミノ酸にわたって存在していた。この遺伝子のDNA配列とこれから予想されるmature enzyme(分子量81，295)のアミノ酸配列は、*K. aerogenes* のDNA塩基配列及びモノアミン酸化酵素のアミノ酸配列とそれぞれ高い相同性があった。これらの結果と酵素活性の解析結果を併せて、本遺伝子がモノアミン酸化酵素($maoA_E$)をコードするものと結論した。

また、*E. coli* の本酵素と他のcopper/topa quinone含有アミン酸化酵素のコファクター部位とのアミノ酸相同性より、topa quinoneへの変換が示唆されているチロシン残基が位置していた。

第四章では、アリールスルファターゼやモノアミン酸化酵素と同様にモノアミン化合物によって誘導合成される30kDaの蛋白質の発見と、その遺伝子解析について述べた。アリールスルファターゼ遺伝子(*atsA*)を含むプラスミド(pAS123)をもつ*K. aerogenes* において、高濃度のチラミンの添加により約30kDaの蛋白質が誘導合成されているのを発見した。この蛋白質はアリールスルファターゼやモノアミン酸化酵素と同様にチラミンに加えてドーパミンやオクトパミンでも誘導された。ディリーション解析により、この蛋白の誘導合成に必須な領域は、*atsBA* オペロンの下流の約2.0kbの断片中に位置することがわかった。この領域のDNA塩基配列より、二つのORF、*moaE* 、*moaF* が見つかった。*moaE* プロモーターはチラミンの添加により活性化され、二つの遺伝子*moaE* 、*moaF* はこのプロモーターからポリシストロニックに転写されていることが示唆された。30kDa蛋白を精製し、そのN末アミノ酸配列を決定したところ、*moaF* 遺伝子のDNA塩基配列から予想されるN末配列と一致していた。また、データベースとのホモロジー検索により、*moaE* 遺伝子産物はチラミンあるいはその代謝産物を酸化還元するこ

とが示唆された。モノアミン化合物による30kDa蛋白の誘導合成は、他のモノアミンレギュロンと同様に正の調節因子*moaR* によって制御されていた。

以上の結果をまとめたのがFig. 5.1. である。

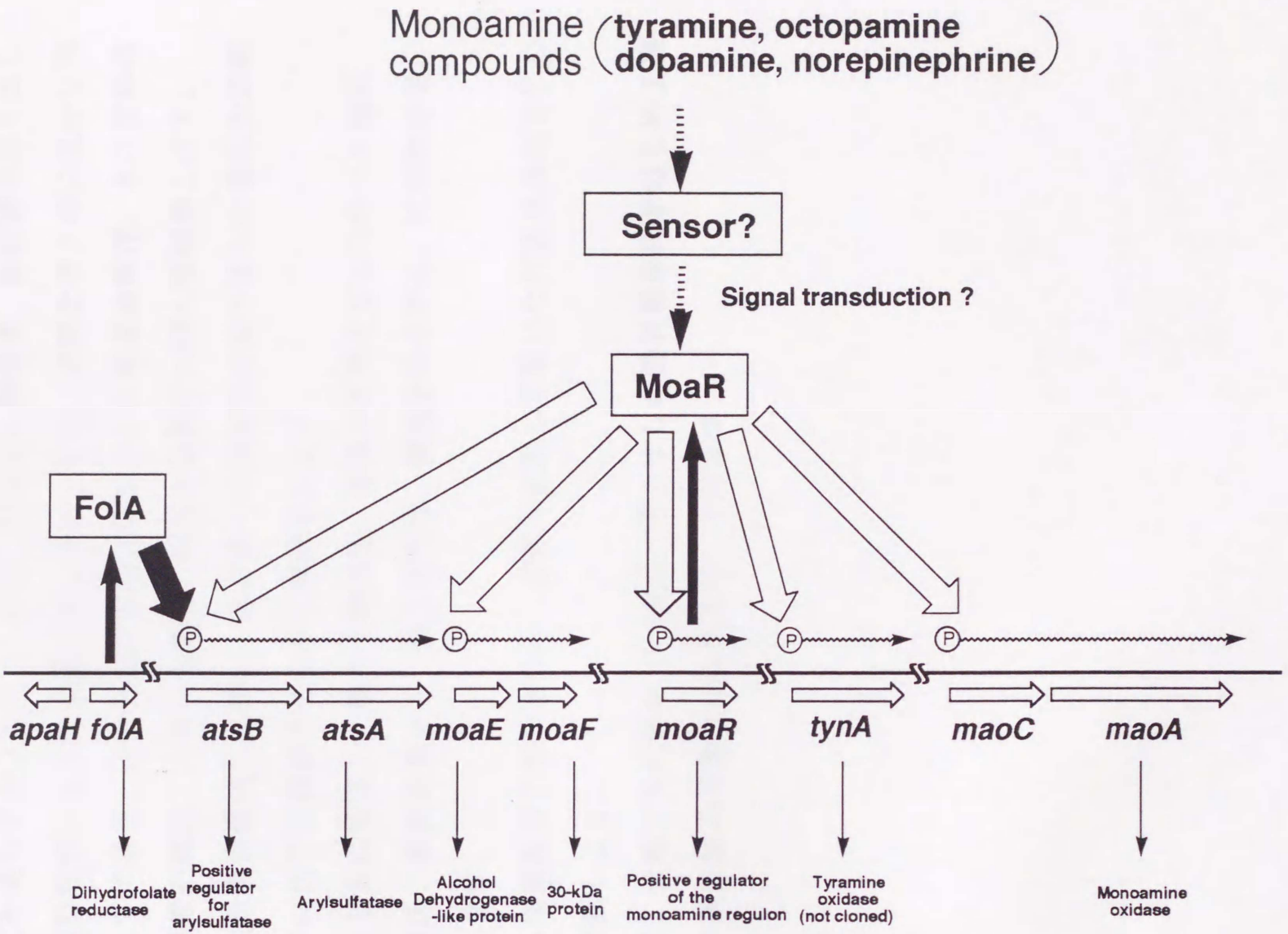


Fig. 5.1. The Monoamine Regulon

謝辞

本研究を遂行するにあたり、終始、懇切なる御指導、御鞭撻を賜りました室岡義勝教授に心から謝意を表します。また、研究を行うのに充分な環境を整えて下さり、かつ本論文査読を賜りました新見治教授、永井史郎教授、宮川都吉教授、大竹久夫教授、西尾尚道教授に深く感謝致します。

実験技術を御教授して頂き、また数々の御助言を賜りました森永力助教授、並びに山下光雄助手に心から感謝致します。

また、ともに研究し、様々な御討論、御協力を頂きました佐々木稔氏、杉野浩幸氏、岩田伸英さん、阪上将司君、横路奈津子さん、片岡敏浩君に感謝致します。

ＤＮＡ塩基配列決定にあたり、装置を操作して頂いた打荻奈美技官に心から感謝致します。

最後に、公私ともに励まし、御協力頂きました野村暢彦氏をはじめ工業微生物学研究室の皆様方にお礼を申し上げます。

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3. **Roh, J., H. Suzuki, H. Azakami, M. Yamashita, Y. Murooka, and H. Kumagai.** Purification, characterization, crystallization, and immune assay of monoamine oxidase from Eschrichia coli. J. Bacteriol. submitted.

4. **Sugino, H., H. Azakami, S. Arai, and Y. Murooka.** Gene targetings in a Klebsiella sp. by fusaric acid selection and the use of temperature sensitive replicon. J. Ferment. Bioeng. submitted.