JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2344–2351
0021-9193/92/072344-08$02.00/0

Vol. 174, No. 7

Cloning and Nucleotide Sequence of a Negative Regulator Gene for *Klebsiella aerogenes* Arylsulfatase Synthesis and Identification of the Gene as *folA*

HIROYUKI AZAKAMI, HIROYUKI SUGINO, AND YOSHIKATSU MUROOKA*

Department of Fermentation Technology, Faculty of Engineering, Hiroshima University, Kagamiyama-1, Higashi-Hiroshima 724, Japan

Received 7 October 1991/Accepted 27 January 1992

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-kb 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-kb 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 for diadenosine tetraphosphatase (*apaH*) in *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 for dihydrofolate reductase (*folA*) in *E. coli*. We detected 30 times more activity of dihydrofolate reductase in the *K. aerogenes* strains carrying the plasmid, which contains the arylsulfatase regulator gene, than in 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.

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 aromatic monoamine compounds. Most studies on the regulation of arylsulfatase biosynthesis have been performed with *Klebsiella aerogenes* because the enzyme is absent from or present at only very low levels in most strains of *Escherichia coli* (2, 10) and is absent from most strains of *Salmonella typhimurium* (17). *K. aerogenes* arylsulfatase is a monomer, with a molecular weight of 47,000 (19, 21), and is located in the periplasmic space of the cell (18). 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$^-$ strains, the enzyme was synthesized constitutively regardless of the source of sulfur (2, 16). These results suggested that sulfate and cysteine acted as the corepressor and that the gene coding for arylsulfatase, *atsA*, was repressed by the *atsR* gene in the presence of these corepressors. The *atsR* mutations were more than 90% cotransducible with *atsA* mutations by PW52 bacteriophage transduction (5).

* Corresponding author.

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 by the action of *maoA* (26) (previously called the tyramine oxidase gene, *tynA*) in *K. aerogenes* (20, 21). The derepression of arylsulfatase synthesis and the synthesis of monoamine oxidase occurred coordinately. The *atsA* and *maoA* genes were mapped on the chromosome of *K. aerogenes* by using F′ episomes from *E. coli* and the transducing phage P1. These genes are linked to *gdhD* and *trp* in the order *atsR-atsA-maoA-gdhD-trp* (18). Recently, the *atsA* and *maoA* genes have been cloned and the nucleotide sequences of regions essential for the expression of arylsulfatase (19) and monoamine oxidase (26a) have been determined. From the analysis of the transcripts, we found that the *ats* operon is composed of the arylsulfatase gene, *atsA*, and the positive regulator gene, *atsB* (19). However, the molecular mechanism of the repression of the *atsA* gene by sulfur-containing compounds has not yet been clarified.

In this report we describe the cloning, characterization, and nucleotide sequence of a negative regulator gene for arylsulfatase from *K. aerogenes*. We have also found that the negative regulator gene codes for 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-kb plasmid which confers resistance to kanamycin (Km^r), was constructed by inserting a 1.1-kb *Pst*I fragment containing the Km^r gene from pUC4K into the *Pst*I site of the plasmid pBR322 (5). Unless other-

TABLE 1. Bacterial strains and their characteristics

Strain	Relevant genotype	Reference
K. aerogenes		
W70	Wild type	12
K304	*tynA13 atsR4*	16
K311	*tynB17 atsR11*	16
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44* λ^- Δ(*lac-proAB*) *relA1* (F', *proAB lacI*^{q}ZΔM15 *traD36*)	29

wise 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, Ohio. [α-^{32}P] dCTP (>650 Ci/mmol) was purchased from ICN Biomedical, Inc., Irvine, Calif. Indoxylsulfate and *p*-nitrophenylsulfate were provided by Sigma Chemical Co., St. Louis, Mo. The other compounds used were standard commercial preparations.

Culture media. The rich medium used was LB (1% polypeptone, 0.5% yeast extract, 0.5% NaCl). The minimal medium used for *K. aerogenes* was K medium (16), consisting of 0.5% carbon source; 0.1% nitrogen source; 0.01% $MgCl_2 \cdot 6H_2O$; 0.001% each NaCl, $MnCl_2 \cdot 4H_2O$, and $FeCl_3 \cdot 6H_2O$; and 0.05 M potassium phosphate buffer (pH 7.2). The media used for *E. coli* strains were supplemented with 1 mM $CaCl_2 \cdot 2H_2O$, threonine and leucine (20 μg/ml each), and thiamine (10 μg/ml). Unless otherwise mentioned, xylose for *K. aerogenes* and succinate for *E. coli* were used as the carbon sources and NH_4Cl and Na_2SO_4 were used as the nitrogen and sulfur sources, respectively.

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). *E. coli* strains were transformed by the method of Hanahan (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 100 mM $CaCl_2$, 50 mM RbCl, and 25 mM LiCl in 100 mM 3-(*N*-morpholino) propanesulfonate (pH 6.5). After incubation for 1 h 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 that complements *atsR* mutations. Chromosomal DNA prepared from *K. aerogenes* W70 by the method of Marmur (14) was partially digested with *Eco*RI or *Bam*HI. Fragments (3 to 10 kb) of the chromosomal DNA were isolated by sucrose gradient centrifugation and ligated to *Eco*RI- or *Bam*HI-cleaved pKI212 by using T4 DNA ligase after treatment with alkaline phosphatase. Cells containing the arylsulfatase constitutive mutation *atsR* were transformed by the $CaCl_2$-heat shock method as described above. Transformants were selected on LB agar plates which contained 50 μg of kanamycin per ml, and Km^r colonies were replicated onto K agar plates with 0.25 mg of indoxylsulfate per ml 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 label and 7-deaza-dGTP instead of dGTP 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 2,000 V and 50 to 55°C, dried on filter paper (3MM; Whatman, Inc., Clifton, N.J.), and visualized by autoradiography with X-Omat AR (Eastman Kodak Co., Rochester, N.Y.) 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 by using a Klett-Summerson colorimeter. Arylsulfatase activity was assayed as described previously (1). One unit of arylsulfatase was defined as the amount of enzyme causing the formation of 1 nmol of *p*-nitrophenol per min at 30°C.

The assay of *K. aerogenes* dihydrofolate reductase was based on that of Stone and Morrison (24, 25) and was performed as follows. Bacteria were grown aerobically at 28°C in K medium. When the culture reached 100 to 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 to 50 μl) was added to 2.5 ml of 50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM NADPH. Aliquots of the mixture (1 ml) 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 A_{340} was monitored by using a JASCO UVIDEC-340 spectrometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan).

Hybridization for gene mapping. The membrane we used was Gene Mapping Membrane (Takara Shuzo Co.), on which immobilized cloned phages covering the *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 a nonradioactive DNA-labeling and detection kit (Boehringer GmbH, Mannheim, Germany), was performed as specified by the manufacturer.

Nucleotide sequence accession number. The sequence discussed in this communication has been deposited with the GenBank/EMBL data bases as no. D10358.

RESULTS

Cloning of the gene that complements *atsR* mutations. 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, 1 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 was designated pASR001.

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

Host strain (genotype)	Plasmid (pASR001)	Sulfur source (3 mM)	Tyramine (3 mM)	Arylsulfatase activity (U/mg of cells)[b]
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 sulfur source indicated, in the presence (+) or absence (−) of tyramine. The cells were harvested when the density of the culture had reached about 100 Klett units.
[b] Values are averages of results from three independent experiments.

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*. The effect of the pAS001 cloned fragment 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, the 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 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 pASR001 was digested with *Eco*RI and *Bam*HI and subcloned into the pKI212 vector. Two plasmids, pASRB1 and pASRB2, which contained the 4.2-kb *Eco*RI-*Bam*HI fragment and the 1.6-kb *Bam*HI-*Eco*RI fragment, respectively, were constructed. The properties of the plasmids were examined by analyzing complementation with the *atsR* mutation. 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 *Eco*RI-*Bam*HI fragment (Fig. 1).

Nucleotide sequence of the negative regulator gene. The complete nucleotide sequence of the 1.6-kb *Eco*RI-*Bam*HI fragment, which carries the regulator gene, was determined (Fig. 2). We found two open reading frames (ORFs) capable of coding for ca. 18- and >29-kDa 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-17 bp-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/mol [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 with a molecular weight of 18,139. We searched the EMBL and SWISS data bases for homologies in the DNA and peptide sequences of the ORFs and other genes. We found that the sequence of the first ORF is 80.0% homologous to the DNA sequence and 90.6% homologous to the amino acid sequence of the *E. coli* dihydrofolate reductase gene, *folA* (Fig. 3). In addition, we found that the second ORF (C-terminal portion) is located counterclockwise to the *folA*-like gene (Fig. 3) and that the DNA and amino acid sequences are 79.1 and 87.6% homologous, respectively, to those of the *E. coli* diadenosine tetraphosphatase gene (*apaH*). From comparison of the amino acid sequence, we found that 20 amino acids from the N terminus of the *K. aerogenes apaH* gene were probably lacking in our clone.

Determination of the essential region for the *atsA* repres-

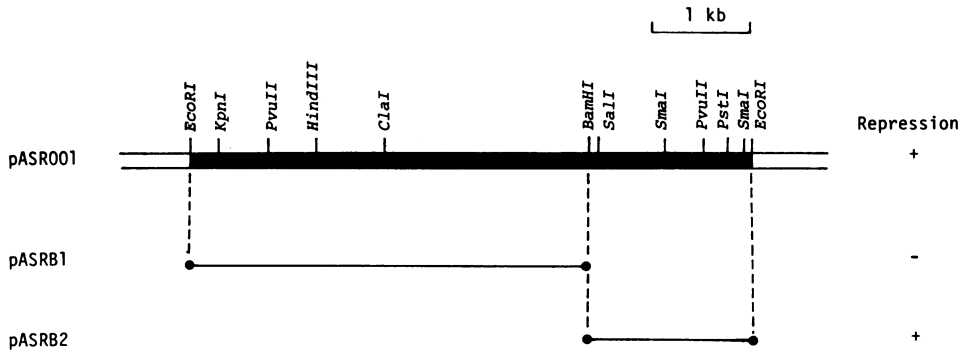

FIG. 1. Restriction endonuclease map of pASR001 and its derivative plasmids. Thick bar, region of DNA cloned; thin bar, DNA remaining in plasmid subclones. Deletion plasmids were used to transform *K. aerogenes* K304 and K311, and cells were assayed for arylsulfatase activity (+, repression level; −,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

sion. 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 *Eco*47III-*Bam*HI 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 that 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. Therefore, we concluded that the first ORF is the *folA* gene of *K. aerogenes*. However, dihydro-

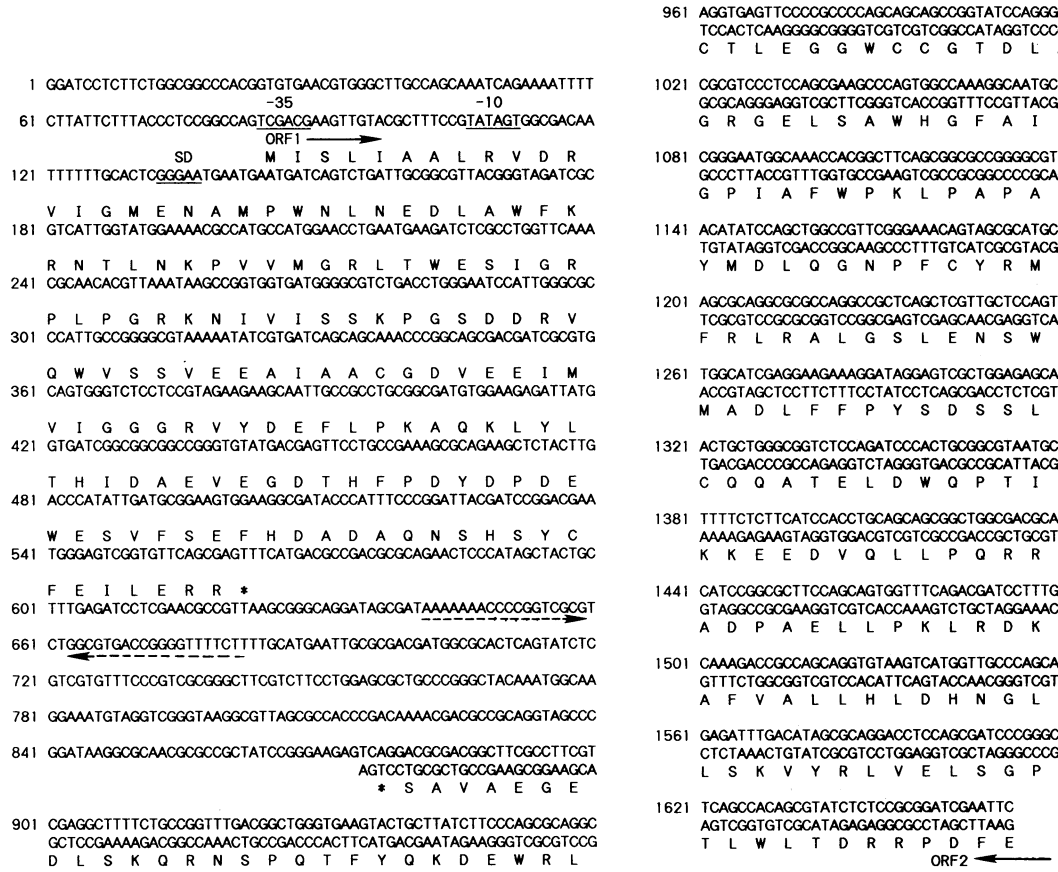

FIG. 2. Nucleotide sequence of the 1.6-kb fragment and the predicted amino acid sequences of the ORF1 and ORF2 genes. The presumptive ribosome-binding site (SD) and possible promoter regions (−10 and −35) are indicated. The arrows show the direction of ORF1 and ORF2. The horizontal dashed arrows show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

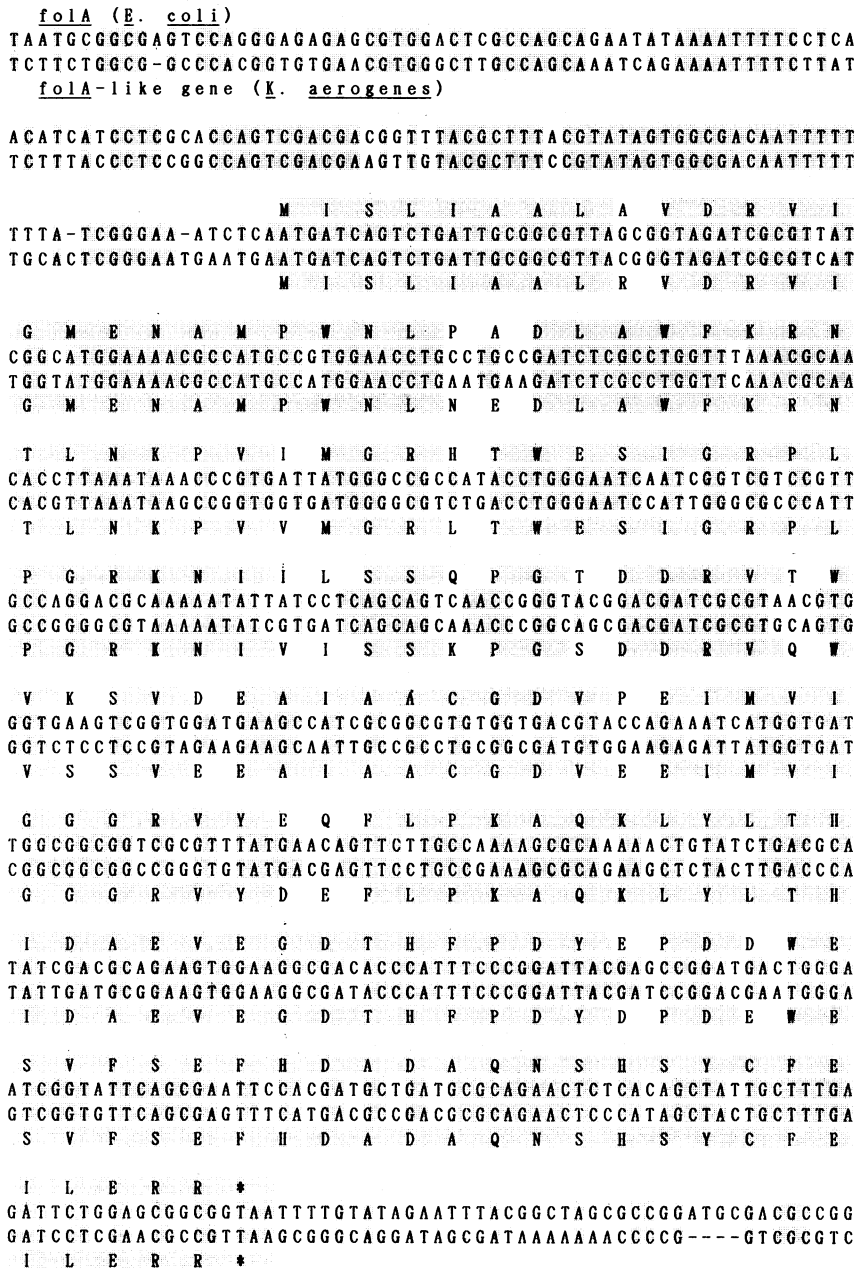

FIG. 3. Comparison of *folA* sequences from *K. aerogenes* and *E. coli*. Identical nucleotides are indicated by the shaded boxes.

folate reductase activities in *atsR* mutant strains 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 ($folA_K$), we mapped the analogous location of the *folA* gene in *E. coli* ($folA_E$). 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 *Sma*I-*Sal*I fragment was isolated from pASRB2 and labeled with a nonradioactive 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 on the *E. coli* genome (Fig. 5). This result is consistent with the previous mapping of the *folA* gene together with the *apaH* gene of the *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 from clone 4A3 with *Eco*RV and inserted into pKI212 vector. The resultant plasmid was designated pEFOL2. Plasmid pEFOL2 was introduced into the *atsR* mutant strains of *K. aerogenes*. It repressed arylsulfatase synthesis in the presence of inorganic sulfate (Table 3). The repression level by *E. coli* $folA_E$ was the same as that by *K. aerogenes* $folA_K$.

From these results, we conclude that the *folA* genes from

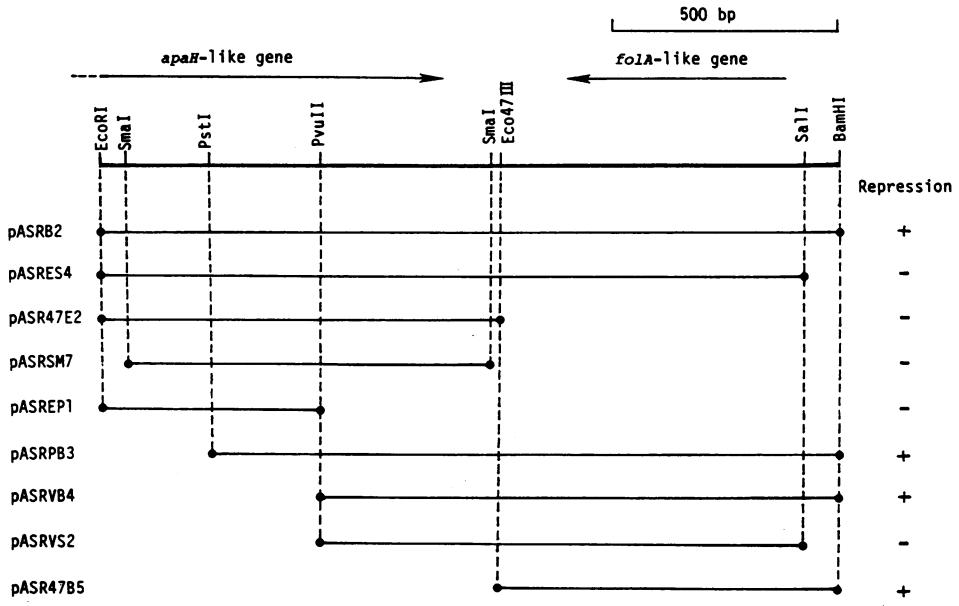

FIG. 4. Restriction map of pASRB2 and its derivative plasmids. Deletion plasmids were used to transform *K. aerogenes* K304 and K311, and cells were assayed for arylsulfatase activity (+, repression level; −, constitutive level). The arrowheads indicate the direction of transcription of the *folA* and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

K. aerogenes and *E. coli* repress otherwise constitutive *atsA* expression in *atsR* mutants.

DISCUSSION

We have cloned a chromosomal fragment of *K. aerogenes* W70, which in multicopy apparently represses arylsulfatase synthesis by *atsR* mutants in the presence of sulfur compounds. Therefore 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 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 in cells grown on sulfate or cysteine as the sole source 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, simple reversal of *atsR*-mediated constitutive expression of *atsA* may occur with low efficiency.

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)[b]	
		Arylsulfatase	Dihydrofolate reductase
W70 (wild type)	None	0.15	0.03
	pASRB2	0.08	0.63
	pEFOL2	0.10	0.52
K304 (*atsR4*)	None	10.4	0.03
	pASRB2	1.60	0.97
	pEFOL2	1.76	0.71
K311 (*atsR11*)	None	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.

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

All results presented here (i.e., deletion analysis of the regulator gene that caused *atsA* repression, sequence homologies of the regulator DNA and amino acid with those of *E. coli folA*, overproduction of dihydrofolate reductase by *K. aerogenes* carrying a 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 wild-type *K. aerogenes* (16), the repression of constitutive *atsR* mutants by the $folA_K$ gene was caused by sulfur and, with the exception of the *atsR4* mutant grown on cysteine, was generally relieved by tyramine (Table 2). These results suggest that 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 by classical genetic analysis that an *E. coli* gene analogous to the *atsA* gene of *K. aerogenes* is located at 27 min on the *E. coli* genome. The *E. coli* gene is judged to be analogous to the *Klebsiella atsA* gene because of common regulatory features discovered in *lac* operon fusions (28). Physical mapping of $folA_K$ with the Kohara bank of the *E. coli* genome shows that the $folA_E$ gene is located at 1 min on the *E. coli* chromosome, far from the location of the *E. coli atsA* homolog just mentioned.

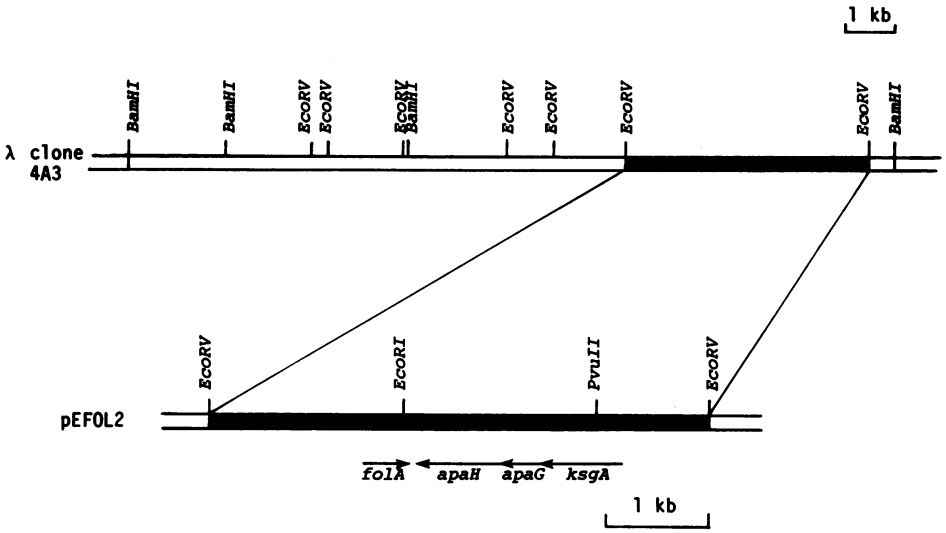

FIG. 5. Restriction map of *E. coli* clone 4A3 (11) and construction of plasmid pEFOL2. The direction of genes was determined by Blanchin-Roland et al. (4).

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 that is cross-reactive with anti-AtsA antibody made to the *Klebsiella* AtsA protein was found (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 in 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* at the level of either 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 (C_1) metabolism and sulfur metabolism.

ACKNOWLEDGMENTS

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

REFERENCES

1. **Adachi, T., Y. Murooka, and T. Harada.** 1973. Derepression of arylsulfatase synthesis in *Aerobacter aerogenes* by tyramine. J. Bacteriol. **116**:19–24.
2. **Adachi, T., Y. Murooka, and T. Harada.** 1975. Regulation of arylsulfatase synthesis by sulfur compounds in *Klebsiella aerogenes*. J. Bacteriol. **121**:29–35.
3. **Adachi, T., H. Okamura, Y. Murooka, and T. Harada.** 1974. Catabolite repression and derepression of arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. **120**:880–885.
4. **Blanchin-Roland, S., S. Blanquet, J. M. Schmitter, and G. Fayat.** 1986. The gene for *Escherichia coli*: diadenosine tetraphosphatase is located immediately clockwise to *folA* and forms an operon with *ksgA*. Mol. Gen. Genet. **205**:515–522.
5. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyreker, and H. W. Boyer.** 1977. Construction and characterization of new vehicles. A multipurpose cloning system. Gene **2**:95–113.
6. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Fitzgerald, J. W.** 1976. Sulfate ester formation and hydrolysis: a potentially important yet often ignored aspect of the sulfur cycle of aerobic soils. Bacteriol. Rev. **40**:698–721.
8. **Hamm-Alvarez, S. F., A. Sancar, and K. V. Rajagopalan.** 1990. The presence and distribution of reduced folates in *Escherichia coli* dihydrofolate reductase mutants. J. Biol. Chem. **265**:9850–9856.
9. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
10. **Harada, T., and B. Spencer.** 1964. Repression and induction of arylsulfatase synthesis in *Aerobacter aerogenes*. Biochem. J. **93**:373–378.
11. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
12. **MacPhee, O. G., I. W. Sutherland, and J. F. Wilkinson.** 1969. Transduction in *Klebsiella*. Nature (London) **221**:475–476.
13. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
14. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J. Mol. Biol. **3**:208–218.
15. **Mizusawa, S., S. Nishimura, and F. Seela.** 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine triphosphate in place of dGTP. Nucleic Acids Res. **14**:1319–1324.
16. **Murooka, Y., T. Adachi, H. Okamura, and T. Harada.** 1977. Genetic control of arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. **130**:74–81.
17. **Murooka, Y., and T. Harada.** 1981. Regulation of derepressed synthesis of arylsulfatase by tyramine oxidase in *Salmonella typhimurium*. J. Bacteriol. **145**:796–802.
18. **Murooka, Y., T. Higashiura, and T. Harada.** 1978. Genetic

mapping of tyramine oxidase and arylsulfatase genes and their regulation in intergeneric hybrids of enteric bacteria. J. Bacteriol. **136:**714–722.

19. **Murooka, Y., K. Ishibashi, M. Yasumoto, M. Sasaki, H. Sugino, H. Azakami, and M. Yamashita.** 1990. A sulfur- and tyramine-regulated *Klebsiella aerogenes* operon containing the arylsulfatase (*atsA*) gene and the *atsB* gene. J. Bacteriol. **172:**2131–2140.
20. **Okamura, H., Y. Murooka, and T. Harada.** 1977. Tyramine oxidase and regulation of arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. **129:**24–31.
21. **Okamura, H., T. Yamada, Y. Murooka, and T. Harada.** 1976. Purification and properties of arylsulfatase of *Klebsiella aerogenes*: identity of the enzyme formed by non-repressed and derepressed synthesis. Agric. Biol. Chem. **40:**2071–2076.
22. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
23. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminator sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
24. **Stone, S. R., and J. F. Morrison.** 1982. Kinetic mechanism of the reaction catalyzed by dihydrofolate reductase from *Escherichia coli*. Biochemistry **21:**3757–3765.
25. **Stone, S. R., and J. F. Morrison.** 1984. Catalytic mechanism of the dihydrofolate reductase reaction as determined by pH studies. Biochemistry **23:**2753–2758.
26. **Sugino, H., K. Ishibashi, M. Sakaue, M. Yamashita, and Y. Murooka.** 1991. Gene cloning of the *maoA* gene and overproduction of a soluble monoamine oxidase from *Klebsiella aerogenes*. Appl. Microbiol. Biotechnol. **35:**606–610.

26a. **Sugino, H., M. Sasaki, H. Azakami, M. Yamashi-Ta, and Y. Murooka.** A monoamine-regulated *Klebsiella aerogenes* operon containing the monoamine oxidase structural gene (*maoA*) and the *maoC* gene. J. Bacteriol., in press.

27. **Yamada, T., Y. Murooka, and T. Harada.** 1978. Comparative immunological studies on arylsulfatase in bacteria of the family *Enterobacteriaceae*: occurrence of arylsulfatase protein regulated by sulfur compounds and tyramine. J. Bacteriol. **133:**536–541.
28. **Yamashita, M., and Y. Murooka.** 1984. Use of *lac* gene fusions to study regulation of tyramine oxidase, which is involved in derepression of latent arylsulfatase in *Escherichia coli*. Agric. Biol. Chem. **48:**1459–1470.
29. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.