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A Sulfur- and Tyramine-Regulated *Klebsiella aerogenes* Operon Containing the Arylsulfatase (*atsA*) Gene and the *atsB* Gene

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The structural gene for arylsulfatase (*atsA*) of *Klebsiella aerogenes* was cloned into a pKI212 vector in *Escherichia coli*. Deletion analysis showed that the *atsA* gene with the promoter region was located within a 3.2-kilobase cloned segment. In *E. coli* cells which carried the plasmid, the synthesis of arylsulfatase was repressed by various sources of sulfur; the repression was relieved, in each case, by tyramine. Transfer of the plasmid into *atsA* or constitutive *atsR* mutant strains of *K. aerogenes* resulted in complementation of *atsA* but not of *atsR*. The nucleotide sequence of the 3.2-kilobase fragment was determined. Two open reading frames, the *atsA* gene and an unknown gene (*atsB*), were found. These are located between a potential promoter and a transcriptional terminator sequence. Deletion analysis suggests that *atsB* is a potential positive factor for the regulation of arylsulfatase. Analysis of the amino acid sequences of the first 13 amino acids from the N terminus of the purified secreted arylsulfatase agrees with that of the nucleotide sequence of *atsA*. The leader peptide extends over 20 amino acids and has the characteristics of a signal sequence. Primer extension mapping of transcripts generated in vivo suggests that the synthesis of mRNA starts at a site 31 or 32 bases upstream from the ATG initiation codon of the *atsB* gene. By Northern (RNA) blot analysis of the transcripts induced by tyramine, we found a 2.7-kilobase transcript which is identical in size to the total sequence of the *atsB* and *atsA* genes. Thus, the *ats* operon is composed of two cistrons and is regulated by sulfur and tyramine.

Arylsulfatase (EC 3.1.6.1) hydrolyzes arylsulfate esters to aryl compounds and inorganic sulfate. The synthesis of arylsulfatase in bacteria is of interest because it is controlled by sulfur compounds and by an aromatic monoamine compound (1). Most studies of the regulation of the synthesis of arylsulfatase have been performed with *Klebsiella aerogenes* because the enzyme is absent from *Escherichia coli* (33). In *K. aerogenes*, the *atsA*-specified arylsulfatase is synthesized constitutively in cells grown with methionine or taurine as the source of sulfur (2). The synthesis of the enzyme is thought to be repressed by the *atsR* gene in the presence of a corepressor derived from inorganic sulfate, cysteine, or related compounds (2, 18). However, no *cis-trans* test of the relationship of the *atsR* gene to the *atsA* gene has been demonstrated. The repression caused by the sulfur-containing compounds is relieved by the addition of tyramine, octopamine, dopamine, or norepinephrine (3, 25). Using a tyramine (monoamine) oxidase-deficient mutant (*tynA*) and tyramine oxidase-constitutive mutants, we showed that the induction of tyramine oxidase by tyramine or by catecholamines resulted in derepression of the *atsA* gene, which is closely linked to *tynA* (19, 24, 25) (Fig. 1). However, the molecular mechanism of derepression of the *atsA* gene by induction of tyramine oxidase has not been clarified. As a step towards understanding the organization of genes in the *ats-tyn* region, we have isolated plasmids that carry the genes involved in the synthesis of arylsulfatase.

This report describes the cloning, characterization, and entire nucleotide sequence of the structural gene for arylsulfatase from *K. aerogenes*. In addition, we present data on the regulation of the expression of the *atsA* gene by sulfur-containing compounds and tyramine, the action of *atsR* in *trans* upon the *atsA* gene, the analysis of transcripts in the *ats* region, and the finding of a potential positive regulator gene.

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MATERIALS AND METHODS

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

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, S1 nuclease, reverse transcriptase, Klenow fragment of DNA polymerase, and calf intestine alkaline phosphatase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan) or Toyobo Co., Ltd. (Osaka, Japan). The M13 sequencing kit with deoxy-7-deazaguanosine triphosphate was purchased from Takara Shuzo Co. *p*-Nitrophenylsulfate (Sigma Chemical Co., St. Louis, Mo.) was recrystallized from aqueous ethanol before use. [α-^{32}P]dCTP and [γ-^{32}P]ATP were purchased from Amersham Corp., Arlington Heights, Ill. The other compounds used were standard commercial preparations.

Plasmids. pKI212, a 5.5-kilobase (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 in the *Pst*I site of the plasmid pBR322 (7). Unless otherwise mentioned, other recombinant plasmids were derivatives of plasmid pKI212. Promoter cloning vector pKK232-8 (8) and pUC4K, a plasmid containing an aminoglycoside 3′-phosphotransferase gene conferring kanamycin resistance, were purchased from Pharmacia, Uppsala, Sweden. pRK290 (10) was provided by D. R. Helinski (University of California at San Diego). pUC119 (constructed by Yanisch-Perron et al. [35]) was purchased from Takara Shuzo Co. pYM006, a plasmid containing the tandem promoters of λp_R and λp_L, the *c*I857-p_{RM}, Km^r, and *galK* genes, was derived from pYM005 (22).

Cloning of the *atsA* gene. Chromosomal DNA prepared from *K. aerogenes* W70 by the method of Marmur (16) was

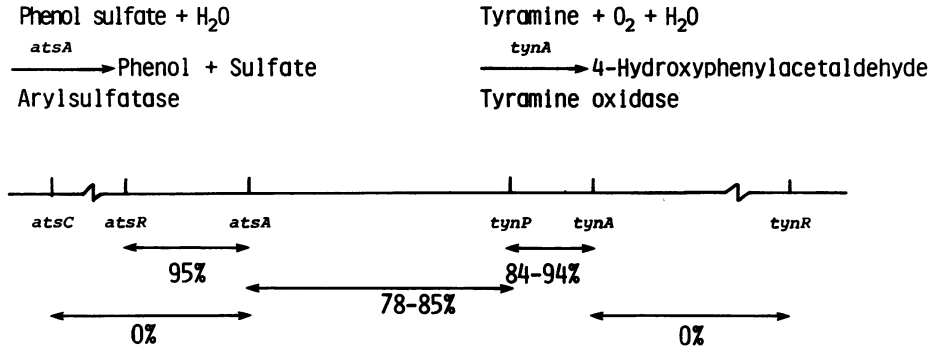

FIG. 1. Diagrams of the relative locations of the *ats* and *tyn* genes (18, 20, 24). Percentages represent cotransduction frequencies by PW52 or P1 phages.

partially digested with *Bam*HI or *Eco*RI. Fragments of between 7 and 20 kb of the chromosomal DNA were isolated by sucrose gradient centrifugation and ligated to *Bam*HI- or *Eco*RI-cleaved pKI212 by T4 DNA ligase after treatment with alkaline phosphatase. *E. coli* C600 or *ats*-deficient cells of *K. aerogenes* were transformed by the $CaCl_2$-heat shock method as described by Davis et al. (9). Transformants were selected on LB (1% polypeptone, 0.5% yeast extract, 0.5% NaCl) agar plates which contained 50 μg of kanamycin per ml. Km^r colonies were replicated onto K medium (20) with 3 mM tyramine and 0.25 mg of indoxylsulfate per ml as an indicator of arylsulfatase activity. Succinate and xylose (0.25%) were used as the carbon sources for *E. coli* and *K. aerogenes*, respectively (34). Ats^+ colonies, which become blue on plates containing indoxylsulfate (2), were selected.

DNA sequence analysis. Sequencing reactions were performed by the M13 dideoxy-chain termination method (28). To eliminate the G-C band compression, we used deoxy-7-deazaguanosine triphosphate instead of deoxyguanosine triphosphate (15). The labeled fragments were separated by polyacrylamide gel electrophoresis under denaturing conditions. The gels were fixed, dried, and exposed overnight to X-ray film.

Synthesis and purification of oligonucleotides. Oligonucleotides for primer extension mapping were synthesized with a Biosearch DNA synthesizer (San Rafael, Calif.). The oligonucleotides were subsequently purified by high-performance liquid chromatography (Japan Spectroscopic Co., Ltd., Tokyo, Japan) with a reverse-phase column (Finepac SIL-C18).

TABLE 1. List of bacterial strains and their characteristics

Strain	Relevant genotype	Source or reference
K. aerogenes		
W70	Wild-type	MacPhee et al. (14)
MK9000	$P1^s$	Streicher et al. (32)
MKN4-1	*atsA41*	Mutagenesis of MK9000
K304	*atsR4*	Murooka et al. (18)
K311	*atsR11*	Murooka et al. (18)
MKN63	*tynA63*	Murooka et al. (20)
E. coli		
C600	*hsdR hsdM thr-1 leu-6 thi-1 atsA*	Ogawa
JM105	*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) (F′ *proAB* $lacI^q$ ΔDM15 *traD36*)	Yanisch-Perron et al. (35)
K12ΔH1Δtrp	M72 Sm^r *lacZ*(Am) *bio-uvrB trpEA2* (λ*N*am7 *N*am53 *c*I857 ΔH1)	Bernard et al. (6)

Preparation of RNA. RNA was isolated from 40 ml of cells in logarithmic growth in K-Na_2SO_4 medium with or without tyramine by the method of Gilman and Chamberlin (11), except that we used proteinase K and proteinase K-treated DNase I at concentrations of 200 μg/ml.

Primer extension mapping. Oligonucleotides complementary to the mRNA were ^{32}P labeled at their 5′ ends with T4 polynucleotide kinase. The labeled primer (10^6 cpm) was mixed with total RNA (100 to 300 μg) in a total volume of 33 μl, and 7 μl of 5× buffer E (250 mM KCl, 25 mM Tris hydrochloride [pH 8.0], 50 mM $MgCl_2$, 5 mM dithiothreitol) was added. The samples were heated at 65°C for 20 min and then cooled slowly to 37°C, and the following solutions were added: 5 μl of a deoxynucleoside triphosphate mixture (5 mM each GTP, ATP, CTP, and TTP) and 1 μl of reverse transcriptase (20 U). After incubation at 42°C for 1.5 h, the samples were hydrolyzed by the addition of 12.5 μl of 0.5 M NaOH, kept at 95°C for 5 min, and then chilled on ice. Subsequently, the NaOH was neutralized by the addition of 12.5 μl of 0.5 M HCl. The reaction mixture was precipitated with ethanol, dried, and dissolved in sequencing dye that contained 80% formamide. Each sample was loaded on a sequencing gel of 8% acrylamide in 8 M urea in a lane next to the corresponding sequence to determine the nucleotide at which transcription began.

S1 nuclease mapping. S1 nuclease mapping was performed as described by Aiba et al. (4). A single strand of DNA was annealed with a synthetic primer DNA, and the DNA probe was prepared by extension from the primer with [α-^{32}P] dCTP, Klenow fragment, and cold deoxynucleoside triphosphate. RNA (100 μg) and 50 ng of the labeled DNA probe (approximately 20,000 cpm) were mixed, lyophilized, dissolved in 30 μl of hybridization buffer (10), heated for 5 min at 90°C, and incubated for 16 h at 42°C. Nonhybridized DNA was digested by the addition of 500 U of S1 nuclease in a final volume of 400 μl and incubation for 1 h at 37°C. Nucleic acids were extracted with phenol, precipitated with ethanol, and analyzed by electrophoresis on 5% acrylamide gels which contained 8 M urea.

Northern (RNA) blot hybridization. Total RNA (20 μg per lane) was fractionated on 1.5% agarose–2.2 M formaldehyde gels as described by Maniatis et al. (15). For molecular weight standards, 18S and 28S rRNA from *Saccharomyces cerevisiae* were used. The RNA was immobilized on Biodyne nylon membrane (Pall BioSupport Co., East Hill, N.Y.) by the capillary method of Southern (31), while the

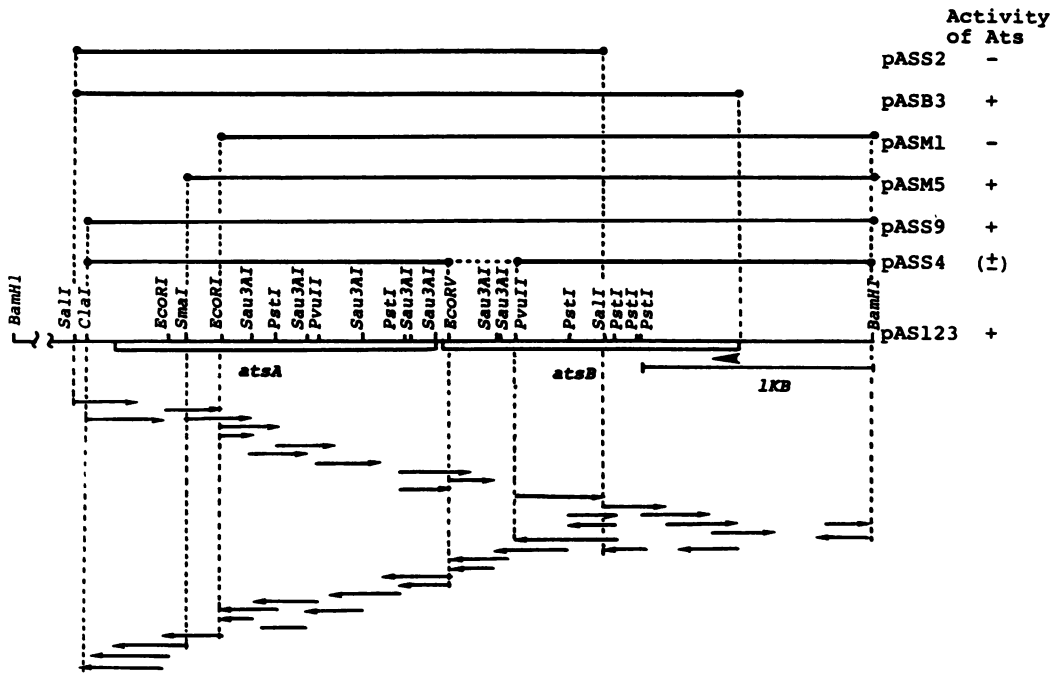

FIG. 2. Restriction endonuclease map of pAS123 and its derivative plasmids and strategy for sequencing. Deletion plasmids were used to transform *E. coli* C600 and *K. aerogenes* MKN4-1, and cells were assayed for arylsulfatase activity. (±), Low level of activity. The arrowhead indicates the direction of transcription of the *atsB* and *atsA* genes. These genes were deduced from the sequence analysis (Fig. 4). The arrows show the lengths and directions of the sequences determined.

hybridization conditions were those recommended by the manufacturers of the membranes. DNA probes were labeled with [α-^{32}P]dCTP and the random-primer DNA labeling system of Nippon Gene Co. (Toyama, Japan).

Purification of arylsulfatase and determination of N-terminal peptide sequence. Arylsulfatase was prepared from the periplasmic fraction of *K. aerogenes* W70(pAS123) and purified to a single protein as described previously (26), except that DEAE-Toyopearl 650S and Toyopearl HW55S (Toso Co., Ltd., Tokyo, Japan) were used instead of DEAE-cellulose and Sephadex G-150, respectively. The enzyme was hydrolyzed in 6 N HCl at 130°C for 4 h, and the amino acid composition of the enzyme was determined with a Hitachi amino acid analyzer (model 835). The amino-terminal sequence of arylsulfatase was determined with a protein sequencer (model 470A; Applied Biosystems, Inc., Foster City, Calif.).

Assay of enzyme activities. Bacteria were grown aerobically at 37°C in K medium. Unless otherwise stated, 0.5% succinate for *E. coli* (34) and 0.5% xylose for *K. aerogenes* (3) and 0.1% NH_4Cl were used as the sources of carbon and nitrogen. Tyramine (3 mM) was used as an inducer of the synthesis of arylsulfatase or as an inducer of tyramine oxidase. Growth was monitored in a Klett-Summerson colorimeter. Arylsulfatase activity was assayed as described previously (1). One unit of arylsulfatase activity was defined as the amount causing formation of 1 nmol of *p*-nitrophenol per min at 30°C. Chloramphenicol acetyltransferase activity was measured as described by Shaw (29). One unit of the enzyme activity was defined as the amount reducing 1 nmol of dithiobisnitrobenzoic acid per min at 37°C.

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

RESULTS

Cloning of the arylsulfatase gene from the *K. aerogenes* chromosome. Since *K. aerogenes* is resistant to ampicillin and sensitive to kanamycin, we constructed the vector pKI212, which has the Km^r gene in a *Pst*I site of pBR322. Chromosomal DNA from *K. aerogenes* W70 was partially digested with *Bam*HI or *Eco*RI. The fragments were ligated with *Bam*HI- or *Eco*RI-cleaved pKI212. The mixture of hybrid DNA molecules was used to transform *E. coli* C600, which has no arylsulfatase activity (20). Among about 5,000 Km^r colonies tested, two Ats$^+$ colonies were obtained by using *Bam*HI-digested fragments, but none were obtained from the *Eco*RI-digested fragments. These Ats$^+$ colonies were recognized by virtue of the fact that colonies stained blue in the presence of indoxylsulfate, a substrate for arylsulfatase, whereas colonies lacking arylsulfatase remained colorless. Plasmid DNAs prepared from the two Ats$^+$ strains were about 7.5 kb in length and were indistinguishable. One of these plasmids, designated pAS123, was used in subsequent experiments.

The transformation of *E. coli* C600 with pAS123 yielded 100% Km^r Ats$^+$ colonies, indicating that the recombinant plasmid was responsible for the ability to produce arylsulfatase. The pAS123 plasmid was further used to transform *K. aerogenes* MKN4-1 (*atsA41*) to Km^r, and all transformants obtained were Ats$^+$.

Localization of the *atsA* gene and the promoter region. A restriction map of the cloned chromosomal fragment was constructed (Fig. 2). A variety of deletion plasmids was constructed by partial or complete digestion with appropriate enzymes. The properties of the plasmids were examined by analyzing the productivity of arylsulfatase in *E. coli* and performing complementation tests with the *atsA* strains of

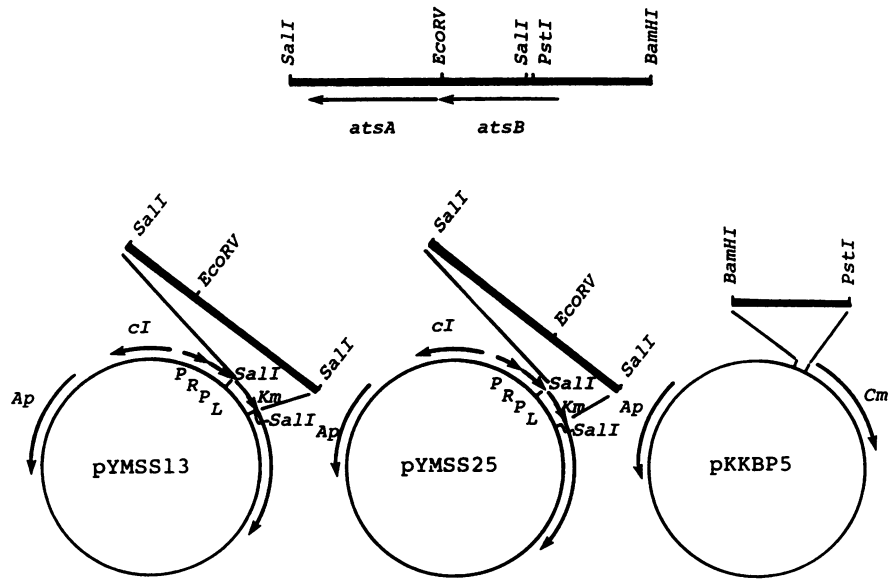

FIG. 3. Construction of *ats'-cat* and $\lambda p_R p_L$-*ats'* fusion genes. The thick lines show segments of the *ats'* gene isolated from pAS123. These fragments were inserted into the promoter probe vector pKK232-8 and expression vector pYM006, which carry the structural gene for *cat* and $\lambda p_R p_L$ promoter, respectively.

K. aerogenes (Fig. 2). The deletion analysis suggests that the *ats* gene is located within the 3.2-kb *Bam*HI-*Cla*I fragment. Since plasmid pAS123 did not complement the *tynA* mutation of *K. aerogenes* MKN63, it lacks a functional *tynA* gene.

To determine the direction of transcription and the promoter region for the *atsA* gene, we tested a 1.0-kb *Bam*HI-*Pst*I fragment inserted into the promoter probe vector pKK232-8, which can express the chloramphenicol acetyltransferase gene (*cat*) when the fragment contains an appropriately oriented promoter (8). All Ap^r Cm^r transformants carried plasmids (pKKBP5) which had the *Bam*HI-*Pst*I fragment (Fig. 3). In these transformants, expression of the *cat* gene was repressed by inorganic sulfate and derepressed by tyramine in every case. These results suggest that a promoter exists in the 1.0-kb *Bam*HI-*Pst*I fragment. Next, a 2.4-kb *Sal*I-*Sal*I fragment was isolated and inserted into plasmid pYM006, in which the $\lambda p_R p_L$ promoter is regulated by the thermolabile *c*I857 repressor. All Ap^r Ats^+ transformants carried the resultant plasmid (pYMSS13), which had the same orientation of the fragment (Fig. 3). In these cells, arylsulfatase was induced at 42°C (but only about fourfold). It was noted that the levels of arylsulfatase in strain K12ΔH1Δtrp which carried pYMSS13 (*atsA*$^+$ Δ*atsB*) were significantly lower (about 1/10) than those in the strain which carried pASS9 (*atsA*$^+$ *atsB*$^+$).

Regulation of the expression of the *atsA* gene in *E. coli* and in *atsA* mutant strains of *K. aerogenes*. Expression of the cloned *atsA* gene in cells of *E. coli* C600(pAS123) and in *K. aerogenes* W70 (wild type) and mutant strain MKN4-1 (*atsA41*), which carries the plasmid pAS123, was tested under various conditions. Table 2 shows arylsulfatase activity in cells grown in synthetic media with inorganic sulfate, cysteine, methionine, or taurine as the sole source of sulfur, both with and without tyramine. No arylsulfatase activity was detected in *E. coli* cells without plasmid. The enzyme was synthesized in *E. coli* only when the cells carried the plasmid pAS123. The synthesis of the enzyme in *E. coli* was repressed when the cells were grown with inorganic sulfate, cysteine, methionine, or taurine in the absence of tyramine. The repression by these sulfur compounds was relieved by the addition of tyramine. The addition of dopamine, octopamine, or norepinephrine in place of tyramine to the

TABLE 2. Synthesis of arylsulfatase in *E. coli* and *K. aerogenes* strains[a]

Host strain (genotype)	Plasmid (pAS123)	Sulfur source (3 mM)	Tyramine (3 mM)	Arylsulfatase activity (U/mg of cells)
E. coli C600	−	Na_2SO_4	+	<0.01
	+	Na_2SO_4	−	1.74
	+	Na_2SO_4	+	6.39
	+	Cysteine	−	1.80
	+	Cysteine	+	7.95
	+	Methionine	−	1.37
	+	Methionine	+	6.31
	+	Taurine	−	1.37
	+	Taurine	+	7.19
K. aerogenes W70 (wild type)	−	Na_2SO_4	−	0.07
	−	Na_2SO_4	+	6.77
	−	Cysteine	−	0.09
	−	Cysteine	+	5.70
	−	Methionine	−	14.6
	−	Methionine	+	15.7
	−	Taurine	−	13.4
	−	Taurine	+	16.8
K. aerogenes MKN4-1 (*atsA41*)	−	Na_2SO_4	+	<0.01
	+	Na_2SO_4	−	5.22
	+	Na_2SO_4	+	19.3
	+	Cysteine	−	5.05
	+	Cysteine	+	27.6
	+	Methionine	−	47.6
	+	Methionine	+	46.5
	+	Taurine	−	49.0
	+	Taurine	+	48.7

[a] *E. coli* and *K. aerogenes* cells were grown in succinate-NH_4Cl and xylose-NH_4Cl media, respectively, with the source of sulfur 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. Effects of sulfur compounds and the *atsR* mutation of *K. aerogenes* or regulation of expression of *atsA*[a]

Host strain (genotype)	Plasmid	Supplement or source of sulfur (mM)	Tyramine (3 mM)	Arylsulfatase activity (U/mg of cells)
MKN4-1 (*atsA41*)		Methionine (3)	+	<0.01
	pARK007	Cysteine (1)	−	0.96
	pARK007	Cysteine (10)	−	0.79
	pARK007	Cysteine (1)	+	10.1
	pARK007	Cysteine (10)	+	9.2
	pARK007	Na_2SO_4 (1)	−	0.87
	pARK007	Na_2SO_4 (10)	−	0.94
	pARK007	Na_2SO_4 (1)	+	9.8
	pARK007	Na_2SO_4 (10)	+	10.1
K304 (*atsA*$^+$ *atsR4*)		Cysteine (10)	−	4.82
		Cysteine (10)	+	5.20
		Na_2SO_4 (10)	−	4.18
		Na_2SO_4 (10)	+	5.35
	pARK007	Cysteine (10)	−	10.3
	pARK007	Cysteine (10)	+	10.0
	pARK007	Na_2SO_4 (10)	−	8.35
	pARK007	Na_2SO_4 (10)	+	10.0
K311 (*atsA*$^+$ *atsR11*)		Cysteine (10)	−	4.69
		Cysteine (10)	+	4.56
		Na_2SO_4 (10)	−	5.90
		Na_2SO_4 (10)	+	6.60
	pARK007	Cysteine (10)	−	9.13
	pARK007	Cysteine (10)	+	9.36
	pARK007	Na_2SO_4 (10)	−	12.6
	pARK007	Na_2SO_4 (10)	+	13.6

[a] The cells were grown in xylose-NH_4Cl medium with the sources of sulfur as indicated, in the presence (+) or absence (−) of tyramine. The cells were harvested and assayed when the density of the culture had reached about 150 Klett units. Values are averages of results from three independent experiments.

various media allowed comparable synthesis of arylsulfatase (data not shown).

In *K. aerogenes* W70, the pattern of regulation was similar, except arylsulfatase was constitutively synthesized when methionine or taurine was used as the sole source of sulfur (Table 2). Plasmid pAS123 complemented the *atsA41* mutation in *K. aerogenes*. The levels of arylsulfatase in the strain which carried pAS123 under derepressed (with tyramine) and constitutive (with methionine) conditions were between two- and threefold higher than those in the wild-type strain W70 (Table 2). However, inorganic sulfate or cysteine weakly repressed the synthesis of the enzyme in the cells which carried a multiple-copy plasmid. This phenomenon may be a result of a limitation of the amount of a regulatory protein or a corepressor.

Effects of a low-copy-number plasmid and the *atsR* mutation on the regulation of the *atsA* gene. To investigate the regulation of the *atsA* gene by the *atsR* mutation, we subcloned the 7.5-kb *Bam*HI chromosomal DNA fragment on plasmid pAS123 into a low-copy-number plasmid, pRK290, which possesses a P1 type replicon and generates only a few copies per host chromosome (10). The resultant plasmid pARK007 was introduced into *K. aerogenes* MKN4-1, and the levels of arylsulfatase in the cells grown with inorganic sulfate or cysteine, with or without tyramine, were tested. Inorganic sulfate and cysteine at a concentration of 1 mM strongly repressed the expression of the *atsA* gene on pARK007 (Table 3). This repression was relieved by tyramine. These results suggest that the *atsR* gene on the chromosome of strain MKN4-1 acts in *trans* upon the *atsA* gene on plasmid pARK007.

In an earlier report, we demonstrated that the *atsR* gene, a negative regulatory gene for the expression of *atsA*, is located near the *atsA* gene (18). Therefore, we tested whether the chromosomal DNA fragment in pARK007 contained the *atsR* gene. The two *atsR* mutations were not complemented by pARK007 (Table 3). No significant difference in the ratios of the extent of repression by sodium sulfate or cysteine at the concentrations of 1 and 10 mM was observed. Thus, 1 mM of a sulfur compound is sufficient to repress the expression of the *atsA* gene on plasmid pARK007.

Nucleotide sequence of *ats*. The complete nucleotide sequence of the *Bam*HI fragment of 3,325 base pairs (bp), which carries the *atsA* gene, was determined (Fig. 4). Unexpectedly, we found two open reading frames (ORFs) capable of coding for about 51- and 45-kilodalton proteins. The first ORF consists of 1,217 bp, with a putative ATG initiation codon at position 452 and a TGA termination codon at position 1667. The putative initiation codon is preceded by a sequence with a high degree of similarity to the −10 and −35 consensus sequence (gTGAtt-19 bp-gAaAAT) and has a potential ribosome-binding site (AGGA) (30). The second ORF contains 1,394 bp with a GTG potential initiation codon at position 1685 and a TGA termination codon at position 3077. The initiation codon, which is located 15 bp downstream from the termination codon of the first ORF, is preceded by the Shine-Dalgarno sequence (AGGA). However, no promoter sequence was found in this region. In the 3′-flanking region of the second ORF, we found a palindromic sequence (ΔG; −43.3 kcal [ca. −181 kJ]/mol) which may act as a transcription terminator (26).

Identification of the product of the *atsA* gene. To identify the product of the *atsA* gene and the processing site that gives rise to the mature protein, we determined the N-terminal sequence of the purified arylsulfatase from the periplasmic fraction of *K. aerogenes* which carried pAS123. The first 13 amino acids were identified as Ala-Gln-Gln-Glu-Arg-Pro-Asn-Val-Ile-Val-Ile-Ile-Ala-. This sequence is identical to that of the directly deduced amino acid sequence from positions 21 to 33 of the second ORF (Fig. 4). The N-terminal region displayed the characteristics expected of a signal peptide, i.e., a basic N-terminal region followed by a central hydrophobic region and a more polar C-terminal region. Therefore, we concluded that the signal peptide is most probably 20 amino acids in length. The translated sequence thus revealed that the precursor protein contains 464 amino acid residues and has a molecular weight of 51,438 and that the mature protein contains 444 amino acids and has a molecular weight of 49,545. The arylsulfatase from the periplasm of *K. aerogenes* W70 was previously shown to be a single polypeptide chain molecule with a molecular weight of about 47,000 (26). The amino acid composition of the second ORF as deduced from the nucleotide sequence was compared with the amino acid composition of the purified arylsulfatase from *K. aerogenes*. Most of the deduced values are identical to the observed values for arylsulfatase (Table 4). Thus, we concluded that the second ORF is *atsA*, which encodes the structural gene for arylsulfatase.

Analysis of a new ORF. The amino acid sequence deduced from the first ORF indicates that the product contains 405 amino acid residues and has a molecular weight of 45,371. The N-terminal region also displayed the characteristics expected of a signal peptide. However, we have not yet determined the N-terminal sequence of the product of the

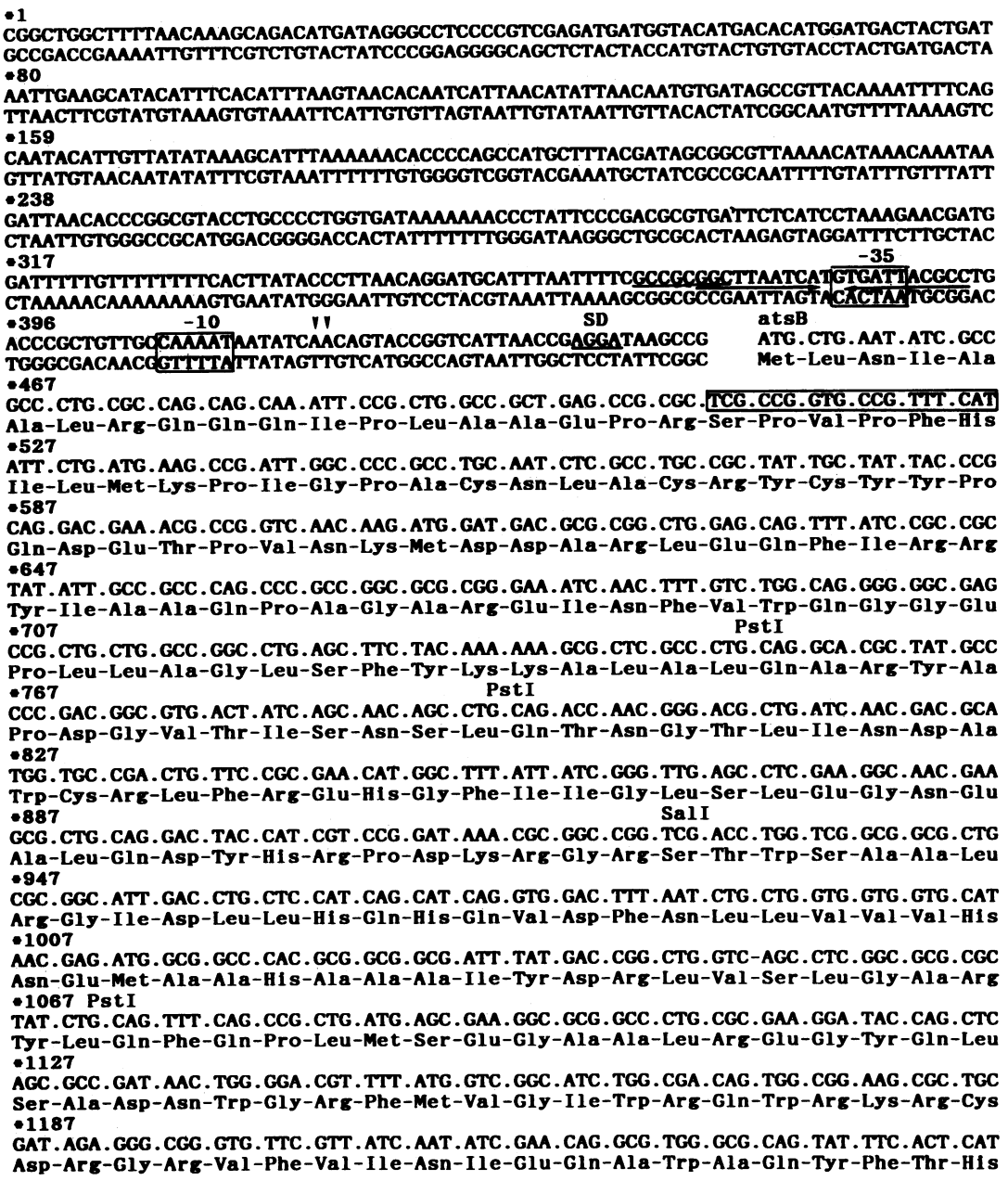

FIG. 4. Nucleotide sequence of the 3.2-kb fragment and the predicted amino acid sequences of *atsB* and *atsA*. The presumptive ribosomal-binding site (SD), possible promoter regions (−10 and −35), and sites of initiation of transcription (▼) are indicated. DNA sequences complementary to the synthetic 18-mer oligonucleotides, used for primer extension and S1 nuclease mapping, are boxed. The horizontal arrows show inverted repeat sequences. The N-terminal amino acid sequence of the secreted arylsulfatase from *K. aerogenes* (▲), determined by the automatic Edman degradation method, is shown. The GenBank/EMBL accession number is M31938.

first ORF. The amino acid composition of this ORF as deduced from the nucleotide sequence showed 14 cysteine residues, whereas no cysteine residue was found in the product of *atsA*.

To clarify the role of the first ORF, we deleted a 0.4-kb *Pvu*II-*Eco*RV fragment from the first ORF and constructed plasmid pASS4 (Fig. 2). The deleted ORF was joined in frame. The levels of arylsulfatase in *K. aerogenes* MKN4-1 which carried pASS4 were about 20% of those in the strain which carried pASS9, irrespective of the presence of tyramine (Table 5). Since the strain MKN4-1 is *atsA* and probably has the complete first ORF, we transferred plasmid pASS4 into *E. coli* C600. No or very little activity of arylsulfatase was found in *E. coli* C600(pASS4). However, no differences in the level of tyramine oxidase were found between pASS9 and pASS4. Thus, the first ORF is a potential positive factor for arylsulfatase. We designated the first ORF the *atsB* gene.

Initiation of transcription of *atsB* and *atsA*. The 5′ termini of the transcripts of *atsB* and *atsA* generated in vivo were

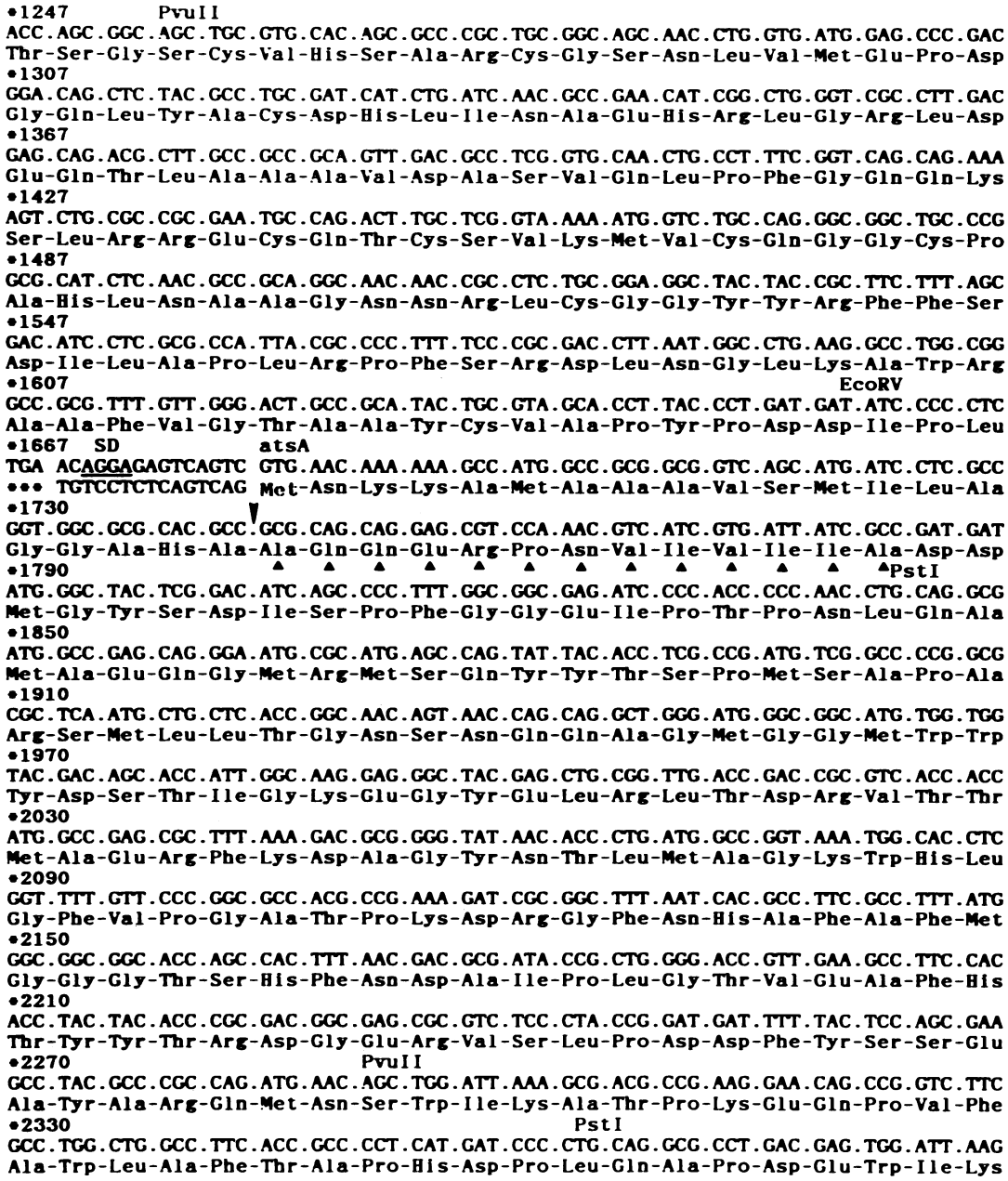

FIG. 4—*Continued.*

determined by the primer extension method with reverse transcriptase. An 18-mer oligonucleotide (TCGCCGGTGC CGTTTCAT) complementary to a sequence in *atsB* was synthesized as a primer (Fig. 4). RNA was isolated from *K. aerogenes* W70 cells cultured in sulfate-containing medium with or without tyramine. Radioactively labeled primer was annealed with RNA and then extended with reverse transcriptase. Two bands which comigrated with chains terminated at thymine were seen (Fig. 5). This corresponded to a message which started at position 420 or 421 on the sequence shown in Fig. 4. The transcripts prepared from cells cultured in the presence of tyramine gave a band more dense than those from cells cultured without tyramine. Cellular RNAs generated in vivo with or without tyramine were also analyzed by S1 nuclease mapping. The S1 nuclease mapping shows the same results as the primer extension mapping does (Fig. 5, lanes 1 and 2). Thus, the transcripts from the *atsB* gene seem to be regulated by sulfate and tyramine.

Northern blot analysis. The sizes of transcripts of the *ats* gene generated in vivo were determined by Northern blot analysis. Total RNA was prepared from *K. aerogenes* W70 cells and from *E. coli* C600(pAS123) cells that were grown in sulfate-containing medium with or without tyramine. A 2.5-kb *SalI-SalI* fragment from the *atsB'-atsA* region was used as the probe. A 2.7-kb transcript was observed with autoradiography (Fig. 6). The transcript was identical in size to that predicted by assuming transcription initiation at

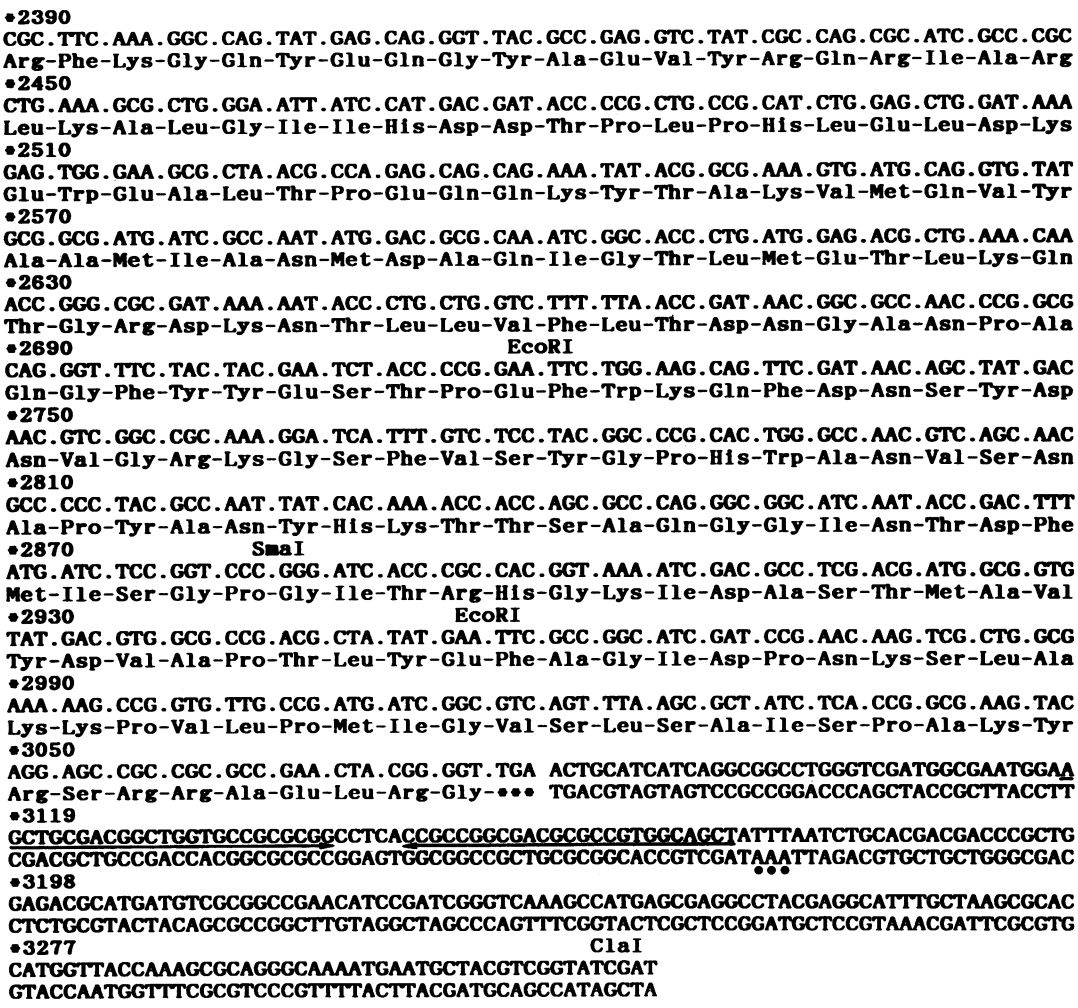

FIG. 4—*Continued.*

position 421 or 422 and termination at about position 3120 (Fig. 4).

DISCUSSION

We have cloned a chromosomal fragment of *K. aerogenes*, which is required for the synthesis of arylsulfatase, into a pKI212 vector in *E. coli*. The transfer of the recombinant plasmid into the arylsulfatase-deficient mutant strain *K. aerogenes* MKN4-1 resulted in complementation of the *atsA* mutation. Deletion analysis of the plasmid showed that all of the sequences required for the synthesis of arylsulfatase are located within a 3.2-kb segment of DNA. The molecular weight of arylsulfatase from *K. aerogenes* was estimated to be between 45,000 and 47,000 (26). The segment of DNA, therefore, is long enough to encode arylsulfatase. The results from gene fusion with the *cat* gene suggest that, within the chromosomal fragment, a promoter which is regulated by sulfur and tyramine exists. Deletion analysis of *atsA* (pASM5) suggests that the C-terminal region (at least 65 amino acid residues) of arylsulfatase is not essential to the activity (Fig. 2).

Within the 3.2-kb DNA segment, we found a new ORF (*atsB*) in addition to the *atsA* gene. The new ORF is located 15 bp upstream of the *atsA* gene. We searched for homologies between the deduced peptide sequences of this ORF and those from other genes but found none. Deletion of the ORF (*atsB*) resulted in a decrease in the level of synthesis of arylsulfatase in *K. aerogenes* MKN4-1 (about 20% of that of the complete gene) irrespective of the presence of tyramine, and no activity was found in *E. coli* C600 (Table 5). This result agreed with the result from the levels of arylsulfatase in *E. coli* K12ΔH1Δtrp which carried pYMSS13 (*atsA*$^+$-Δ*atsB*) (Fig. 3). Thus, the product of the *atsB* gene is most probably involved in the regulation of arylsulfatase but not in the expression of *tynA*.

The deletion analysis, results from gene fusions between $\lambda p_R p_L$ and *atsB'-atsA*, nucleotide sequence, and data from primer extension and S1 nuclease mapping and Northern blot analysis suggest that the *atsA* gene is transcribed from the promoter region upstream of the *atsB* gene and that the *atsB* and *atsA* genes are part of an operon. We have designated this operon the *ats* operon. The transcript from the *ats* operon was repressed by sulfur compounds and derepressed by tyramine or catecholamines. Thus, the regulation of the synthesis of arylsulfatase by sulfur compounds and tyramine or catecholamines occurs at the level of transcription.

The degree of repression of the synthesis of arylsulfatase

TABLE 4. Comparison of the amino acid composition of AtsB and mature AtsA proteins predicted from the DNA sequence with that obtained from protein analysis of mature arylsulfatase

Amino acid	Mature AtsA protein deduced from DNA sequence (%)		Mature arylsulfatase (%)	AtsB protein deduced from DNA sequence (%)
Asp	5.62			4.93
		} 9.89	10.40	
Asn	4.27			4.43
Thr	6.74		6.59	2.46
Ser	5.84		4.11	4.68
Glu	4.94			3.94
		} 9.66	11.56	
Gln	4.72			6.16
Pro	6.29		6.82	5.17
Gly	8.54		8.43	7.39
Ala	10.11		10.24	11.82
Cys	0.00		ND[a]	3.45
Val	4.04		4.98	4.93
Met	4.27		4.02	1.97
Ile	4.72		3.93	4.68
Leu	6.07		6.85	10.59
Tyr	5.39		5.08	3.94
Phe	4.27		4.19	3.94
Lys	4.94		5.40	2.22
His	2.25		2.60	2.96
Trp	2.02		ND	1.97
Arg	4.72		4.80	8.13

[a] ND, Not determined.

by sulfur compounds, in particular by methionine and taurine, varied in different strains of bacteria. Methionine and taurine caused little, if any, repression of synthesis in *K. aerogenes* (2) but strongly repressed synthesis of the enzyme in *Salmonella typhimurium* (13, 19) and *Serratia marcescens* (23). However, this repression is also relieved by the addition of tyramine or catecholamines. In *E. coli*, we also found that the expression of the *atsA* gene was repressed by methionine and taurine and that the synthesis of the enzyme was derepressed by tyramine (Table 2). These organism-specific differences in degree of repression of arylsulfatase by methionine and taurine may be due to differences in the metabolic conversion of these compounds into the actual corepressor for the *atsR* gene.

Although *atsR* mutations, which permit constitutive synthesis of arylsulfatase, are more than 90% cotransducible with *atsA* mutations by PW52 bacteriophage transduction (18), the gene (which complements the *atsR* mutation) was not contained in the chromosomal fragment of approximately 4 kb upstream from the *atsA* gene. However, the *atsR* gene on the chromosome acts in *trans* upon the *atsA* gene on a low-copy-number plasmid (Table 3). The observation of a low level of repression of arylsulfatase in the cells which carried a multiple-copy plasmid suggests that the amount of the *atsR* protein is necessary to repress the expression of the *atsA* gene. Recently, we have cloned the *atsR* gene, and the *atsR* gene also acts in *trans* upon the *atsA* gene on the chromosome (H. Azakami and Y. Murooka, unpublished results). The fragment containing the *atsR* gene

TABLE 5. Effect of the *atsB* gene on the expression of *atsA*[a]

Host strain	Plasmid	Tyramine	Arylsulfatase activity (U/mg of cells)
K. aerogenes MKN4-1 (*atsA41*)		−	<0.01
		+	<0.01
	pASS9	−	2.17
	pASS9	+	13.7
	pASS4	−	0.42
	pASS4	+	3.23
E. coli C600		−	<0.01
		+	<0.01
	pASS9	−	1.87
	pASS9	+	4.05
	pASS4	−	<0.01
	pASS4	+	0.01

[a] The cells were grown in K medium with 3 mM sodium sulfate, in the presence (+) or absence (−) of tyramine. Levels of arylsulfatase synthesis were determined as described in Table 2, footnote *a*.

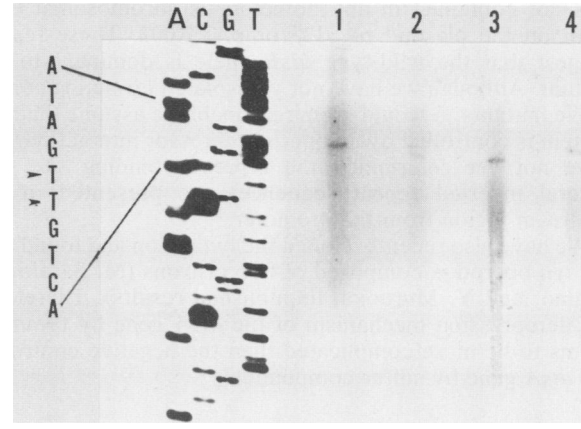

FIG. 5. Primer extension and S1 nuclease mapping of transcripts made in vivo from the *ats* promoter region. RNA was isolated from logarithmically growing cells of *K. aerogenes* W70 grown in K-Na_2SO_4 medium with (lanes 1 and 3) or without (lanes 2 and 4) tyramine. The arrowheads indicate the probable in vivo transcription initiation site for the promoter. Lanes 1 and 2, S1 nuclease mapping; lanes 3 and 4, primer extension mapping.

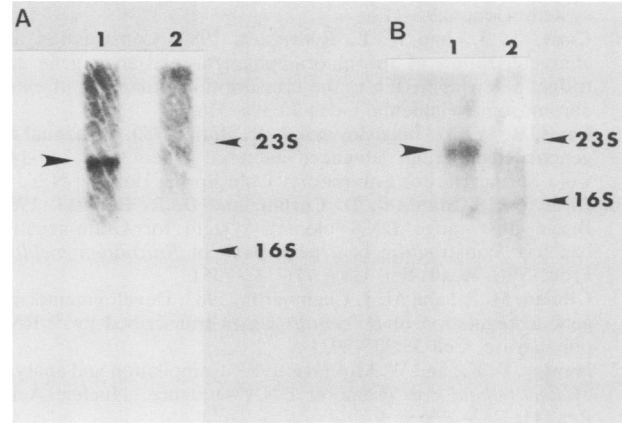

FIG. 6. Northern blot analysis of transcripts from the *ats* region. RNA was isolated from logarithmically growing cells of *K. aerogenes* W70 (A) or *E. coli* C600(pAS123) (B) grown in K-Na_2SO_4 medium with (lanes 1) or without (lanes 2) tyramine. A 2.5-kb *SalI-SalI* fragment, which carried *atsB'-atsA*, was used as the probe. The 16S (1.54 kb) and 23S (2.90 kb) ribosomal RNAs (arrowheads on right of each panel) from *K. aerogenes* were fixed with 1 M acetate and stained with 0.2% methylene blue. The arrowheads on the left of each panel indicate a 2.7-kb transcript.

was not contained in any homologous chromosomal fragment on the plasmid pAS123 or pARK007. These results suggest that the wild-type *atsR* allele is dominant to the mutant. Although we have not yet isolated *cis*-acting constitutive mutants, it would seem reasonable to assume that this system is controlled by an operon-repressor interaction. We have not yet determined the repressor-binding site, but several inverted repeat sequences are presented in the upstream region from the promoter.

We have also recently cloned the *tyn* region and found that the *tyn* operon is composed of two cistrons (M. Sasaki, H. Sugino, and Y. Murooka, unpublished results). Therefore, the derepression mechanism of the *atsA* gene by tyramine seems to be more complicated than the negative control of the *atsA* gene by sulfur compounds.

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