JOURNAL OF BACTERIOLOGY, Apr. 1977, p. 74-81
Vol. 130, No. 1
Copyright © 1977 American Society for Microbiology
Printed in U.S.A.

Genetic Control of Arylsulfatase Synthesis in *Klebsiella aerogenes*

YOSHIKATSU MUROOKA,* TOSHIRO ADACHI,[1] HARUKI OKAMURA,[2] AND TOKUYA HARADA

Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka, Japan

Received for publication 16 September 1976

It was shown that at least four genes are specifically responsible for arylsulfatase synthesis in *Klebsiella aerogenes*. Mutations at chromosome site *atsA* result in enzymatically inactive arylsulfatase. Mutants showing constitutive synthesis of arylsulfatase (*atsR*) were isolated by using inorganic sulfate or cysteine as the sulfur source. Another mutation in which repression of arylsulfatase by inorganic sulfate or cysteine could not be relieved by tyramine was determined by genetic analysis to be on the tyramine oxidase gene (*tyn*). This site was distinguished from the *atsC* mutation site, which is probably concerned with the action or synthesis of corepressors of arylsulfatase synthesis. Genetic analysis with transducing phage PW52 showed that the order of mutation sites was *atsC-atsR-atsA-tynA-tynB*. On the basis of these results and previous physiological findings, we propose a new model for regulation of arylsulfatase synthesis.

Early work on the synthesis of arylsulfatase by *Aerobacter aerogenes* showed that the enzyme is synthesized when the cells are grown on a medium containing methionine or taurine as the sulfur source and that the enzyme synthesis is repressed when inorganic sulfate or cysteine is present as the sole sulfur source or together with taurine or methionine (6, 14). Addition of tyramine, dopamine, octopamine, or norepinephrine to the medium relieves the repression of arylsulfatase synthesis caused by inorganic sulfate or cysteine (1, 5). However, the lack of a suitable genetic exchange system in *A. aerogenes* ATCC 9621 encouraged us to shift our studies to a closely related strain, *Klebsiella aerogenes* W70. The regulation of arylsulfatase synthesis in *K. aerogenes* W70 is quite similar to that in *A. aerogenes* (3). We also demonstrated that the derepression of arylsulfatase synthesis by tyramine is related to the synthesis of tyramine oxidase (11).

Genetic analysis of mutant strains should provide information about the regulation of arylsulfatase and tyramine oxidase syntheses in *K. aerogenes*. This was accomplished by using the transducing phage PW52.

MATERIALS AND METHODS

Bacterial strains. The derivatives of *K. aerogenes* W70 used in this work are listed in Table 1. Transducing phage PW52, described by MacPhee et al. (9), was provided by S. Yamagishi, Chiba University, Japan.

Media. The rich media used were nutrient broth (Difco Laboratories, Detroit, Mich.) and LB (13) containing (per liter): tryptone (Difco), 10 g; yeast extract, 5 g; and NaCl, 5 g. The minimal medium contained 0.5% carbon source, 0.1% nitrogen source, 0.05 M potassium phosphate buffer (pH 7.2), 0.01% $MgCl_2 \cdot 6H_2O$, and 1 mM sulfur compounds. Unless otherwise mentioned, xylose and NH_4Cl were used as carbon and nitrogen sources, respectively. The sulfur compounds used are described for the individual experiments. The plates used for determining the Tyn^- phenotype contained minimal medium with tyramine as the sole source of nitrogen and were supplemented with leucine (50 μg/ml) when required.

Isolation of mutants. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was used as a mutagenic agent, as described by Adelberg et al. (4). The mutagenized cells were treated with penicillin G (3,000 U/ml) for 3 to 5 h to achieve enrichment of auxotrophs. For isolation of mutants in which control of arylsulfatase synthesis was altered, cells were spread on agar plates of selective minimal medium containing 1 mM sodium sulfate with or without tyramine (1 mM). Two screening techniques were used as reported earlier (2).

Preparation of phage lysates and assay. *K. aerogenes* strains were grown aerobically by reciprocal shaking at 30°C in 100-ml volumes of nutrient broth medium. When cultures reached 120 Klett units, PW52 phage was added at a multiplicity of infection of 0.1. Cultures were incubated at 30°C on a rotary shaker at 250 rpm. When lysis was evident (after 12 to 16 h), most of the cells were removed by centrifugation at 8,000 × *g* for 10 min; the rest were removed by passing the lysate through a membrane filter (pore size, 0.45 μm; Toyokagaku Co., Tokyo,

[1] Present address: National Institutes of Health, Bethesda, MD 20014.

[2] Present address: Institute for Microbial Diseases, Osaka University, Osaka, Japan.

Japan). The resulting lysates had titers ranging from 1×10^{10} to 5×10^{10} infectious particles per ml. The confluent lysis method of Rosner (15) was also used for preparation of small volumes of phage lysates, and the resulting preparations usually had titers of about 10^{11} infectious particles per ml.

Phage titers were determined as follows. *K. aerogenes* W70 was grown in LB medium to a density of 5 $\times 10^8$ bacteria per ml. Then, 0.1-ml portions of appropriate dilutions of the lysate were mixed with 0.2 ml of strain W70 (10^9/ml), 4 ml of LB soft agar was added, and the mixture was poured onto fresh LB plates and incubated at 30°C for 12 to 16 h.

Transduction. Samples (0.2 ml each) of late-exponential-phase cultures of recipient cells in nutrient broth medium (2.5×10^9/ml) were suspended in PW52 phage lysate prepared from the donor strain at a multiplicity of infection of 2.0. The phage were allowed to adsorb for 20 min at 30°C, the mixture was centrifuged at 5,000 $\times$ *g*, and the cells were suspended in saline. Then, 0.1-ml samples of cell suspension were spread on selective plates and incubated at 30°C until colonies appeared. When tyramine was used as the nitrogen source, colonies generally appeared after 36 to 48 h. For detection of arylsulfatase-positive or -negative transductants, *p*-nitrocatechol sulfate or indoxyl sulfate was used, as described previously (2).

Assay of arylsulfatase. Arylsulfatase activity was assayed as described previously (1). One unit of activity was defined as the amount causing formation of 1 nmol of *p*-nitrophenol per min at 30°C.

Assay of tyramine oxidase. Tyramine oxidase was assayed by the method of Snyder and Hendley (16). The reaction mixture (1.8 ml) contained 2 μmol of [^{14}C]tyramine hydrochloride (0.5 μCi/μmol), 0.05 M potassium phosphate buffer (pH 7.4), and crude enzyme extract. One unit of enzyme activity was defined as the amount metabolizing 1 pmol of tyramine per min at 30°C.

Protein determination. Protein was determined by the method of Lowry et al. (8), with bovine serum albumin as a standard.

Assay of tyramine uptake. After three doublings, cells were harvested, washed twice with 0.05 M phosphate buffer (pH 7.2) at 5°C, and resuspended in the same volume of cold buffer solution. Uptake of [^{3}H]tyramine was measured as described previously (3).

Chemicals. [^{14}C]- and [^{3}H]tyramine hydrochloride were purchased from the Radiochemical Centre, Amersham, England. *p*-Nitrophenyl sulfate, obtained from Sigma Chemical Co., St. Louis, Mo., was recrystallized from aqueous ethanol before use. The other compounds used were standard commercial preparations.

RESULTS

Genetic linkage of arylsulfatase and tyramine oxidase genes. We found by transduction with PW52 phage that the arylsulfatase gene is closely linked to the tyramine oxidase gene (*tyn*). This makes it possible to analyze the structural or regulatory genes for arylsulfatase by selection of tyramine oxidase-positive colonies that can grow with tyramine as the sole source of nitrogen.

We isolated most of the arylsulfatase-nega-

TABLE 1. *K. aerogenes strains and their characteristics*

Strain	Relevant genotype[a]	Comments
W70	Wild type	MacPhee et al. (9)
K11	*leu-1*	Okamura et al. (11)
K13	*tynA13*	Adachi et al. (3)
K17	*tynB17*	Adachi et al. (3)
K47	*tynA47*	Mutagenesis of W70
K50	*tynA50*	Mutagenesis of W70
K51	*tynA51*	Mutagenesis of W70
K58	*tyn-58*	Mutagenesis of W70
K63	*tynA63*	Mutagenesis of W70
K65	*tyn-65*	Mutagenesis of W70
K66	*tynA66*	Mutagenesis of W70
K019	*tynA019*[b]	Okamura et al. (11)
K110	*tynA110*[b] *leu-1*	Okamura et al. (11)
K111	*atsA111 leu-1*	Mutagenesis of K11
K113	*tynA113*[b] *leu-1*	Okamura et al. (11)
K114	*atsA114 leu-1*	Mutagenesis of K11
K119	*atsA119 leu-1*	Mutagenesis of K11
K171	*tynB17 leu-2*	Mutagenesis of K17
K174	*tynB17 atsA174 leu-2*	Mutagenesis of K17
K304	*tynA13 atsR4*	Mutagenesis of K13
K307	*tynA13 atsR7*	Mutagenesis of K13
K311	*tynB17 atsR11*	Mutagenesis of K17
K312	*tynB17 atsR12*	Mutagenesis of K17
K313	*tynB17 atsR13*	Mutagenesis of K17
K314	*tynB17 atsR14*	Mutagenesis of K17
K315	*tynB17 atsR15*	Mutagenesis of K17
K601	*tynA13 atsA1*	Adachi et al. (3)
K602	*tynA13 tyn-602*[b]	Okamura et al. (11)
K603	*tynA13 atsA3*	Mutagenesis of K13
K604	*tynA13 atsA4*	Mutagenesis of K13
K605	*tynA13 atsA5*	Mutagenesis of K13
K607	*tynA13 tyn-607*	Mutagenesis of K13
K608	*tynA13 atsA8*	Mutagenesis of K13
K609	*tynA13 tyn-609*	Mutagenesis of K13
K611	*tynA13 tyn-611*[b]	Okamura et al. (11)
K615	*tynA13 atsA15*	Mutagenesis of K13
K616	*tynA13 tyn-616*	Mutagenesis of K13
K617	*tynA13 tyn-617*[b]	Okamura et al. (11)
K1721	*tynB17 atsC21 leu-2*	Mutagenesis of K171
K1725	*tynB17 atsC25 leu-2*	Mutagenesis of K171
K1737	*tynB17 atsC37 leu-2*	Mutagenesis of K171
K1739	*tynB17 atsA39 leu-2*	Mutagenesis of K171
K1763	*tynB17 atsC63 leu-2*	Mutagenesis of K171
K1764	*tynB17 atsC64 leu-2*	Mutagenesis of K171
K3041	*atsR4*	Revertant of K304
K3071	*atsR7*	Revertant of K307
K6015	*atsA1*	Revertant of K601
K6031	*atsA3*	Revertant of K603
K6041	*atsA4*	Revertant of K604
K6052	*atsA5*	Revertant of K605
K6083	*atsA8*	Revertant of K608
K6153	*atsA15*	Revertant of K615

[a] *tyn*$^-$, Tyramine oxidase defective, previously designated as *mao*$^-$ (3); the differences between *tynA* and *tynB* are described in the text. *atsA*, Defect of structural gene for arylsulfatase. *atsC*, Tyramine can release arylsulfatase repression caused by cysteine but not by inorganic sulfate. *leu*$^-$, Requirement for leucine. *atsR*, Constitutive synthesis of arylsulfatase in the presence of inorganic sulfate or cysteine.

[b] Previously designated as *atsT*, representing lack of derepression of arylsulfatase synthesis by tyramine.

tive mutants (Ats⁻) from *K. aerogenes* strain K13 or K171, both of which are deficient in tyramine oxidase, an enzyme involved in tyramine degradation (3). Some mutant strains were isolated from K11, which is a leucine-requiring strain derived from W70. Arylsulfatase synthesis occurred in strains K13, K171, and K11 grown in xylose-methionine medium. When cells were grown with inorganic sulfate, arylsulfatase synthesis was repressed. This repression in the parent strains was relieved by the addition of tyramine, as reported previously (3). However, little or no arylsulfatase synthesis was observed in the *atsA* mutant strains under any culture conditions.

The linkage of the various *ats* mutations to *tyn* was determined by transducing phage grown on the wild type into the Ats^- Tyn^- recipients. Tyn^+ recombinants were selected and replica-plated onto minimal medium-tyramine plates containing indoxyl sulfate to determine the percentage of Ats^+ Tyn^+ recombinants (Table 2). Results with *p*-nitrocatechol sulfate or indoxyl sulfate as the indicator were identical, but the former was more sensitive. All of the *atsA* alleles were more than 78% cotransducible with *tyn-13*, whereas *atsA* was not closely linked to *tyn-17*. The results make it difficult to assume that both are mutations in the same gene. These two *tyn* genes are tentatively denoted as *tynA* and *tynB*, respectively.

TABLE 2. *Frequency of atsA alleles among Tyn^+ transductants*

Strain[a]		No. scored	Cotransduction frequency (%)
Donor	Recipient		
W70 (+)	K601 (*tynA13*)	1,246	85.2
K17 (*tynB17*)	K601 (*tynA13*)	400	85.5
K70 (+)	K603 (*tynA13*)	527	84.1
W70 (+)	K604 (*tynA13*)	400	78.3
W70 (+)	K605 (*tynA13*)	431	82.1
W70 (+)	K608 (*tynA13*)	285	88.1
W70 (+)	K615 (*tynA13*)	214	78.1
K174 (*tynB17*)	K13 (*tynA13*)	396	81.8
K1739 (*tynB17*)	K13 (*tynA13*)	1,589	84.1
K111 (+)	K13 (*tynA13*)	111	89.2
K114 (+)	K13 (*tynA13*)	618	80.3
K119 (+)	K13 (*tynA13*)	343	85.4
W70 (+)	K174 (*tynB17*)	400	0.3
K1739 (*tynB17*)	K47 (*tyn-47*)	154	33.3
K111 (+)	K50 (*tyn-50*)	170	82.4
K1739 (*tynB17*)	K51 (*tyn-51*)	464	39.4
K1739 (*tynB17*)	K58 (*tyn-58*)	146	4.1
K1739 (*tynB17*)	K63 (*tyn-63*)	262	93.5
K1739 (*tynB17*)	K65 (*tyn-65*)	227	16.3
K1739 (*tynB17*)	K66 (*tyn-66*)	918	54.0
K119 (+)	K019 (*tyn-019*)	545	88.2
K119 (+)	K110 (*tyn-110*)	308	78.4
K119 (+)	K113 (*tyn-113*)	143	81.9

[a] Genotype is shown only for *tyn* allele numbers; a plus (+) represents tyn^+.

Therefore, we isolated several mutants incapable of growth on tyramine as the sole source of nitrogen. All of these mutant strains had lost tyramine oxidase activity (Table 3). The arylsulfatase activities of the mutants were normal when the cells were grown with methionine or sodium sulfate plus tyramine. Table 2 shows that the *tyn* mutations listed in Table 3 are cotransducible with *atsA*. It seems that *tyn-63* is closest to *atsA*, with *tynA13* in between *tyn-63* and the cluster containing *tyn-50, tyn-66, tyn-51*, and *tyn-47*. These mutations are probably located at the *tynA* site. Unfortunately, the cotransduction technique was not satisfactory for determining the order of closely linked mutations or for deciding whether *tyn-65* and *tyn-58* are in the same cluster with *tynB17*. Strains K019, K110, and K113, which were derived from wild-type W70 and in which tyramine does not derepress arylsulfatase synthesis, were deficient in tyramine oxidase as described below. Mutation sites of these strains are closely linked to *tynA13*.

Fine-structure analysis of *atsA*. A more-detailed analysis of the mutation order in *atsA* was achieved by reciprocal crosses in which phage from an $AtsA^-$ strain was introduced into a $TynA^-$ $AtsA^-$ strain. Tyn^+ recombinants were selected and analyzed for the Ats^+ phenotype. Controls were performed to check for spontaneous reversion, and crosses were made in which a given *ats* mutation was crossed with itself to determine whether transduction increased the mutation rate. Spontaneous reversion (less than 10^{-8} in all of the mutant strains used) did not interfere with evaluation of the

TABLE 3. *Levels of arylsulfatase and tyramine oxidase in mutants unable to grow on tyramine as the nitrogen source*[a]

Strain	Tyramine oxidase activity (U/mg of protein)	Arylsulfatase activity (U/mg of cells)		
		Methionine (1 mM)	Na_2SO_4 (1 mM)	Na_2SO_4 (1 mM) + tyramine (1 mM)
W70	724	20.5	2.1	98.8
K13	<1	16.8	1.8	95.5
K17	5	10.8	1.5	59.0
K47	<1	16.9	1.9	67.2
K50	4	26.4	2.4	68.0
K51	<1	17.2	3.3	72.0
K58	<1	32.4	2.1	62.5
K63	<1	18.3	4.4	47.0
K65	52	18.3	3.4	105
K66	1	17.1	1.7	59.0

[a] Cells were harvested after approximately three doublings.

crosses. The results of definitive crosses are shown in Table 4. Crosses number 1 and 2 suggest the order: *atsA1*- *atsA5*- *tynA13*, the 1.3% presumably representing quadruple crossovers. Reciprocal crosses 3 to 12 likewise indicated the order *atsA15-A1-A4-A3-A5-tynA13*. No order could be deduced from crosses 13 and 14. The percentage of Ats$^+$ Tyn$^+$ recombinants was less than 7%, indicating that mutation sites were closely linked.

We also attempted to confirm the location of the loci by obtaining all possible combinations of reciprocal crosses. Crosses 15 to 30 also strongly support the above-mentioned order. The large number of Tyn$^+$ Ats$^+$ recombinants in crosses 19 and 25 indicates that *atsA15* is relatively far from *atsA5* and *atsA8*. Together the results of these reciprocal crosses (crosses number 1 to 30) establish the orders *atsA15-atsA1-atsA4-atsA3-atsA8* and *atsA5-tynA13*. Mutations of *atsA5* and *atsA8* probably have the same mutation site or a very close linkage.

Genetic determinant of arylsulfatase-constitutive mutations. Mutants producing aryl-

TABLE 4. *Analysis of fine structure of atsA*

Number	Relevant genotype		Tyn$^+$ transductants analyzed (no.)	Ats$^+$ (%)	Probable order of mutations
	Donor (*tyn*$^+$)	Recipient (*tynA13*)			
1	K6015 *atsA1*	K605 *atsA5*	1,101	5.6	*atsA1-atsA5-tyn*
2	K6052 *atsA5*	K601 *atsA1*	1,416	1.3	
3	K6031 *atsA3*	K605 *atsA5*	3,657	0.7	*atsA3-atsA5-tyn*
4	K6052 *atsA5*	K603 *atsA3*	2,057	0.0	
5	K6031 *atsA3*	K601 *atsA1*	2,126	2.6	*atsA1-atsA3-tyn*
6	K6015 *atsA1*	K603 *atsA3*	2,164	5.6	
7	K6015 *atsA1*	K604 *atsA4*	1,031	6.0	*atsA1-atsA4-tyn*
8	K6041 *atsA4*	K601 *atsA1*	2,519	2.8	
9	K6031 *atsA3*	K604 *atsA4*	1,732	0.0	*atsA4-atsA3-tyn*
10	K6041 *atsA4*	K603 *atsA3*	4,067	1.4	
11	K6153 *atsA15*	K601 *atsA1*	1,484	5.7	*atsA15-atsA1-tyn*
12	K6015 *atsA1*	K615 *atsA15*	1,350	3.2	
13	K6083 *atsA8*	K605 *atsA5*	2,224	0.0	(*atsA8-atsA5*)*-tyn*
14	K6052 *atsA5*	K608 *atsA8*	2,776	0.0	
15	K6031 *atsA3*	K608 *atsA8*	3,029	0.8	*atsA3-atsA8-tyn*
16	K6083 *atsA8*	K603 *atsA3*	2,078	0.0	
17	K6041 *atsA4*	K605 *atsA5*	2,153	1.8	*atsA4-atsA5-tyn*
18	K6052 *atsA5*	K604 *atsA4*	1,186	0.1	
19	K6153 *atsA15*	K605 *atsA5*	1,500	7.1	*atsA15-atsA5-tyn*
20	K6052 *atsA5*	K615 *atsA15*	629	1.4	
21	K6041 *atsA4*	K608 *atsA8*	3,410	1.1	*atsA4-atsA8-tyn*
22	K6083 *atsA8*	K604 *atsA4*	1,022	0.1	
23	K6015 *atsA1*	K608 *atsA8*	3,150	4.4	*atsA1-atsA8-tyn*
24	K6083 *atsA8*	K601 *atsA1*	882	1.0	
25	K6153 *atsA15*	K608 *atsA8*	1,580	6.3	*atsA15-atsA8-tyn*
26	K6083 *atsA8*	K615 *atsA15*	799	0.6	
27	K6153 *atsA15*	K603 *atsA3*	1,928	3.3	*atsA15-atsA3-tyn*
28	K6031 *atsA3*	K615 *atsA15*	1,521	1.5	
29	K6153 *atsA15*	K604 *atsA4*	827	3.4	*atsA15-atsA4-tyn*
30	K6041 *atsA4*	K615 *atsA15*	2,021	1.7	

sulfatase in the presence of inorganic sulfate or cysteine were isolated from strains K17 and K13. The mutants listed in Table 5 had arylsulfatase activity in the presence of Na_2SO_4, cysteine, or methionine as the sole sulfur source. This type of mutant, in which arylsulfatase is synthesized constitutively, is called AtsR⁻. The arylsulfatase level in mutants grown with methionine was higher than that of the wild-type strain, suggesting that the partial repression of arylsulfatase synthesis by methionine or its metabolites was completely relieved by mutations in *atsR*, which might be the gene for the repressor molecule for arylsulfatase.

Linkage of the various *atsR* mutations to *tyn* was determined by transducing phage grown on each AtsR⁻ strain into a TynA⁻ AtsA⁻ recipient. Tyn⁺ recombinants were selected, and AtsA⁺ colonies were replica-plated on xylose-sodium sulfate plates without tyramine to determine the percentage of AtsR⁻ AtsA⁺ recombinants. Table 6 shows that these *atsR* mutations were all more than 95% cotransducible with *atsA* mutations. A more-detailed analysis of the mutation order was obtained by three-point crosses in which phage from an AtsR⁻ or Ats⁻ strain was introduced into a TynA⁻ AtsA⁻ or TynA⁻ AtsR⁻ strain. Tyn⁺ recombinants were selected and analyzed for the Ats⁺ phenotype (Table 7). Crosses number 1 and 2 suggest the order *atsR4- atsA1- tynA13*, the 1.6% presumably representing quadruple crossovers. This order was also supported by crosses number 3 and 4. Crosses 5 and 6 likewise showed that *atsR7* is on the side of *atsA1* distal to *tynA13*. The small number of Tyn⁺ AtsA⁺ AtsR⁺ recombinants indicates that *atsR* is relatively close to *atsA*.

Demonstration of two control sites for derepressed synthesis of arylsulfatase. In a previous paper (11), we suggested that the derepressed synthesis of arylsulfatase by tyramine was due to the synthesis of tyramine oxidase, because the regulation patterns of arylsulfatase and tyramine oxidase were similar and because mutants in which tyramine did not derepress arylsulfatase synthesis were deficient in tyramine oxidase. Most of the regulatory mutants that we isolated did not show derepression of arylsulfatase synthesis by tyramine, and these mutations were previously called AtsT⁻. The levels of arylsulfate and tyramine oxidase in these mutant strains are summarized in Table 8. The mutants were distinguished from AtsA⁻ strains by the fact that they showed high enzyme activity in a medium containing methionine as the sulfur source. It can be seen that tyramine oxidase activity was lost in strains K602, K607, K609, K611, and K617, as well as in three mutant strains (K110, K113, and K019) derived from wild-type W70, as reported previ-

TABLE 5. *Arylsulfatase levels in AtsR strains*[a]

Strain	Arylsulfatase activity (U/mg of cells)		
	Methionine (1 mM)	Na_2SO_4 (1 mM)	Cysteine (1 mM)
K311	43.1	87.0	49.5
K312	60.7	76.9	54.1
K313	61.4	62.1	48.8
K314	63.1	59.3	57.7
K315	111.3	87.8	79.3
K304	52.3	50.6	70.0
K307	24.0	15.2	15.5

[a] Cells were harvested after 20 h of cultivation.

TABLE 6. *Genetic linkage of atsR and atsA to tynA by transductions with strain K601 (tynA13 atsA1) as recipient*

Donor	Tyn⁺ recombinants analyzed (no.)	*atsR⁻/atsA⁺*	%
K3041 (*atsR4*)	997	784/830	94.5
K3071 (*atsR7*)	430	356/370	96.2
K311 (*atsR11*)	1,816	1,596/1,597	98.4
K312 (*atsR12*	1,118	854/864	98.8
K313 (*atsR13*)	1,439	1,171/1,194	98.1
K314 (*atsR14*)	1,524	1,238/1,248	99.2
K315 (*atsR15*)	1,605	1,314/1,348	97.4

TABLE 7. *Order of atsR⁻ mutations deduced by three-point crosses*

No.	Relevant genotype		Tyn⁺ transductants analyzed (no.)	*atsA⁺/atsR⁺* (%)	Probable order
	Donor (*tyn⁺*)	Recipient (*tynA13*)			
1	K3041 *atsR4*	K601 *atsA1*	997	5.0	*atsR4-atsA1-tynA13*
2	K6015 *atsA1*	K304 *atsR4*	745	1.6	
3	K3041 *atsR4*	K604 *atsA4*	419	4.5	*atsR4-atsA4-tynA13*
4	K6041 *atsA4*	K304 *atsR4*	1,000	1.2	
5	K3071 *atsR7*	K601 *atsA1*	832	4.8	*atsR7-atsA1-tynA13*
6	K6015 *atsA1*	K307 *atsR7*	705	1.3	

TABLE 8. *Arylsulfatase and tyramine oxidase levels in various regulatory mutants*[a]

Strain	Arylsulfatase activity[b] (U/mg of cells)					Tyramine oxidase[c] (U/mg of protein)
	Methionine	Na_2SO_4	Cysteine	Na_2SO_4 + tyramine	Cysteine + tyramine	
W70	16.8	0.3	0.3	55.3	42.3	724
K13	15.6	0.6	0.5	68.2	56.6	<1
K171	9.7	0.7	0.8	57.2	63.8	15
K602	5.9	0.2	0.2	1.0	0.3	<1
K607	12.9	0.3	0.3	1.0	0.7	<1
K609	12.6	0.7	0.4	0.7	0.3	<1
K611	11.7	0.8	0.7	0.6	0.3	<1
K616	7.7	1.1	0.7	0.3	1.0	<1
K617	18.2	1.4	0.4	0.4	0.7	<1
K110	9.8	0.6	0.1	0.6	0.3	<1
K113	7.9	0.6	<0.1	0.6	0.3	<1
K019	11.3	0.6	0.4	0.5	0.4	<1
K1721	18.3	0.4	0.2	2.1	11.9	21
K1725	7.4	0.3	0.2	0.9	6.3	18
K1737	0.6	0.4	0.2	1.2	18.6	16
K1763	2.7	0.1	0.3	0.1	4.8	32
K1764	0.3	0.2	0.2	<0.1	8.6	1

[a] Cells were harvested after aproximately three doublings.
[b] A 1 mM concentration of sulfur source or tyramine was used.
[c] A 1 mM concentration of tyramine was added as an inducer.

ously (11). It is possible that these strains could not take up tyramine, but all the strains listed in Table 8 appeared to take up tyramine into the cells at rates similar to that of the wild type (about 4 nmol of tyramine/min per mg of cells).

To clarify the relationship between the regulatory mutation and the *tyn* gene, transductions were made from the wild-type strain to regulatory mutants. Tyn^+ recombinants were selected on xylose-sodium sulfate plates in the presence of tyramine as the nitrogen source so that the percentage of Ats^+ Tyn^+ recombinants could be calculated. All of the Tyn^+ recombinants were also Ats^+, although the recipient strains are Ats^- in Na_2SO_4- tyramine medium (Table 9). These results suggest that the regulatory site concerned with derepressing arylsulfatase synthesis by tyramine is in the *tyn* gene. We also isolated Tyn^+ revertants from strains K110 and K113. All of these revertants had tyramine oxidase activity and could synthesize arylsulfatase in the presence of tyramine and sodium sulfate. However, we failed to isolate Tyn^+ revertants from strains K602, K607, K609, K611, K617, and K019. These strains may contain multiple mutations or deletions in the *tyn* gene, whereas K110 and K113 have a single mutation in the *tynA* gene (Table 2). Thus, arylsulfatase derepression is regulated by at least the *tynA* gene.

Strains K1721, K1725, K1737, and K1763, which were derived from strain K171, could synthesize arylsulfatase in a medium containing cysteine as the sulfur source in the presence of tyramine, although tyramine could not relieve repression of arylsulfatase by sodium sulfate. The phenotypic characteristics of the mutation that caused synthesis of arylsulfatase in the presence of cysteine and tyramine, and repressed synthesis of the enzyme in the presence of inorganic sulfate and tyramine, is denoted here as $AtsC^-$.

TABLE 9. *Genetic analysis of the regulatory mutants incapable of derepressed synthesis of arylsulfatase by tyramine*

Donor	Recipient	Tyn^+ transductants analyzed (no.)	Ats^+ recombinants in Na_2SO_4-tyramine medium (no.)	Linkage (%)
W70	K602	456	456	100
K17	K602	599	599	100
W70	K607	320	319	99.7
W70	K609	160	160	100
W70	K611	400	400	100
W70	K616	171	171	100
W70	K617	139	139	100
W70	K110	175	175	100
W70	K113	94	94	100
W70	K019	505	505	100
K1721	K13	628	628	0
K1725	K13	1,004	1,004	0
K1737	K13	166	166	0
K1763	K13	334	334	0
K1764	K13	1,570	1,570	0

Linkage of *atsC* mutations to *tynA13* was

analyzed by transducing phage grown on an AtsC⁻ strain into Tyn⁻ recipients. The *atsC* gene was not cotransduced with *tynA* by phage PW52 (Table 9). Figure 1 summarizes the data obtained from these genetic analyses.

DISCUSSION

Arylsulfatase occurs widely in animal tissues and in microorganisms. Its widespread distribution suggests that it has a rather fundamental function, but little is known about this. It is important to know the function of genes responsible for arylsulfatase synthesis. In *K. aerogenes*, some genes are probably responsible for metabolic control of aryl and sulfur compounds, whereas others specify the structure of arylsulfatase. The arylsulfatase of *K. aerogenes* is a monomer with a molecular weight of 47,000 (12). It has been demonstrated that purified preparations of arylsulfatase produced under nonrepressing conditions with methionine as the sulfur source and under derepressing conditions in the presence of tyramine and inorganic sulfate or cysteine have identical proteins (12). Thus, a single gene probably determines the primary structure of the enzyme molecule.

We conclude that the structural gene for arylsulfatase is at the *atsA* locus. This conclusion is based on the observations that none of the mutants isolated as arylsulfatase-negative strains showed any enzymatic activity under any culture conditions and that these Ats^- mutations were all approximately 85% cotransducible with *tynA13*. It is particularly interesting that the tyramine oxidase gene (*tyn*) is closely linked to the structural gene for arylsulfatase. The *tynB* gene is distinguished from the *tynA* in the cotransduction frequency but not in their phenotypic characteristics. Examination of the fine structure of the *atsA* site (Table 4) shows that mutations in the *tyn*-distal and *tyn*-proximal segments coincided fairly well with the results of two-point crosses (Table 2). Therefore, the mutation sites of other arylsulfatase-negative mutants, (*ats-39, ats-174, ats-114,* and *ats-119*) are probably located at the *atsA* site.

Arylsulfatase synthesis now appears to be regulated by at least three different genes,

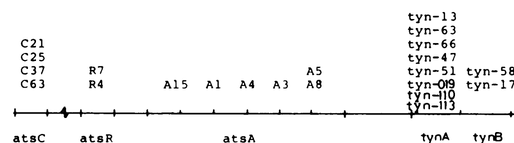

FIG. 1. *Map of the ats and tyn sites of K. aerogenes. Distances do not represent actual cotransduction percentages.*

atsR, tynA, and *atsC,* which have been distinguished from each other by their phenotypic characteristics and by genetic analyses.

One of the sites for arylsulfatase regulation is represented by the *atsR* mutation. This mutation is constitutive for arylsulfatase under all cultivation conditions. The arylsulfatase synthesis of strain K311 was insensitive to sulfite, sulfide, and thiosulfate, in addition to sulfate and cysteine (2). Furthermore, the uptakes of inorganic sulfate and cysteine by this strain were not significantly different from those of the parent strain K17. Genetic analysis showed that *atsR* is closely linked to *atsA* on the side distal to *tynA*. These results are consistent with the present concept of the operon: i.e., expression of the arylsulfatase gene is regulated by a typical repressor-corepressor system, like the operons in amino acid biosyntheses (7, 17). However, we have not been able to assign regulator or operator functions because of our inability to generate diploids.

A second site apparently involved in regulation of derepressed synthesis of arylsulfatase caused by tyramine was represented previously by the $AtsT^-$ mutation (3). Strains with this mutation could not utilize tyramine or octopamine as the nitrogen source, and they lacked tyramine oxidase (12). Moreover, Tyn^+ revertants from strains K110 and K113 had the ability to derepress arylsulfatase synthesis in the presence of tyramine and inorganic sulfate or cysteine. These facts and the results of genetic analysis by transduction, i.e., that all the Tyn^+ transductants acquired the ability to produce arylsulfatase in sodium sulfate-tyramine medium, strongly suggest that the mutation site (AtsT) exists in the *tynA* gene. Since most Tyn^- strains had the ability to derepress synthesis of arylsulfatase and regulatory mutants were difficult to obtain by mutagenesis from the wild-type strain, mutants incapable of derepressed synthesis of arylsulfatase may have a mutation resulting in loss of the ability to transcribe the tyramine oxidase gene, such as loss of a promoter locus or a nonsense mutation in the tyramine oxidase operon, or they may have a defect in tyramine oxidase at the regulation site for derepression of arylsulfatase but not at the active site for the substrate of tyramine oxidase. These results support the previous conclusion that the derepression of arylsulfatase caused by tyramine is dependent upon expression of the tyramine oxidase gene or a protein concerned with the tyramine oxidase gene, and that the activity of tyramine oxidase is not necessary for derepression of arylsulfatase (11).

The other site for arylsulfatase regulation was represented by *atsC* in which tyramine

could release arylsulfatase repression caused by cysteine but not by inorganic sulfate. It has been shown that the repressions of arylsulfatase synthesis by inorganic sulfate and by cysteine are different and that there are at least two independent functional corepressors of arylsulfatase synthesis in *K. aerogenes* (2). Mutation of *atsC* probably changes something concerned with sulfur metabolism, resulting in alteration of the corepressor of arylsulfatase, although we do not yet know about the real corepressor of arylsulfatase synthesis.

Our proposed scheme of the regulatory mechanisms is shown in Fig. 2. Arylsulfate ester is hydrolyzed to an aryl compound and inorganic sulfate by *atsA*-specified arylsulfatase. The inactive repressor (R1) coded by *atsR* is activated (R2) by a corepressor, which comes from inorganic sulfate or cysteine and is governed by *atsC*. On the other hand, tyramine oxidase is specified by *tyn* and induced by specific monoamine compounds, such as tyramine, which could be one of the products of the action of arylsulfatase on arylsulfate ester (10,12). Monoamine compounds such as tyramine, dopamine, octopamine, or norepinephrine are oxidized to hydroxyphenylacetaldehyde compounds and ammonium ions. The ammonium effect is independent of the function of cyclic adenosine 3',5'-monophosphate (10). The site required for derepression of arylsulfatase is the *tynA* gene. Expression of the *tynA* gene results in derepression of arylsulfatase synthesis. Details of the derepression mechanisms involving the *tynA* gene are still unknown. It is possible that tyramine oxidase or a related protein(s) decreases the intracellular level of corepressor of arylsulfatase synthesis or inhibits the function of repressor. Not all of the *ats* or *tyn* genes may yet have been identified. For instance, there may be several genes, such as *atsS* (2), required for the production or action of the corepressor.

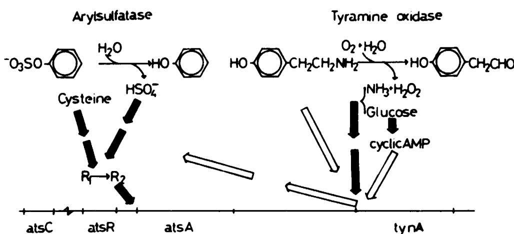

FIG. 2. *Model for the regulation of arylsulfatase and tyramine oxidase. For explanation of this model, see text. The regulation of tyramine oxidase is based on reference 10. Symbols:* ⇦, *positive control;* ⬅, *negative control.*

ACKNOWLEDGMENTS

We thank T. Iizima and L. Johnson of the National Institutes of Health for help in preparing this manuscript.

This work was supported by grants 876066 and 056123 from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

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. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. 18:788-795.
5. **Harada, T.** 1957. Phenolsulphatase synthesis with tyramine as an inducer by *Aerobacter aerogenes*. Bull. Agric. Chem. Soc. Jpn. 21:267-273.
6. **Harada, T., and B. Spencer.** 1964. Repression and induction of arylsulfatase synthesis in *Aerobacter aerogenes*. Biochem. J. 93:373-378.
7. **Jacoby, G. A., and L. Gorini.** 1969. A unitary account of the repression mechanism of arginine biosynthesis in *E. coli*. I. The genetic evidence. J. Mol. Biol. 39:73-87.
8. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
9. **MacPhee, D. G., I. W. Sutherland, and J. F. Wilkinson.** 1969. Transduction in *Klebsiella*. Nature (London) 221:475-476.
10. **Okamura, H., Y. Murooka, and T. Harada.** 1976. Regulation of tyramine oxidase synthesis in *Klebsiella aerogenes*. J. Bacteriol. 127:24-31.
11. **Okamura, H., Y. Murooka, and T. Harada.** 1977. Tyramine oxidase and regulation of arylsulfatase synthesis in *Klebsiella aerogenes*. J. Bacteriol. 129:59-65.
12. **Okamura, H., T. Yamada, Y. Murooka, and T. Harada.** 1976. Purification and properties of arylsulfatase of *Klebsiella aerogenes:* identity of the enzymes formed by non-repressed and de-repressed synthesis. Agric. Biol. Chem. 40:2071-2076.
13. **Prival, M. J., and B. Magasanik.** 1971. Resistance to catabolite repression of histidase and proline oxidase during nitrogen-limited growth of *K. aerogenes*. J. Biol. Chem. 246:6288-6296.
14. **Rammler, D. H., C. Grado, and L. R. Fowler.** 1964. Sulfur metabolism of *Aerobacter aerogenes*. I. A repressible sulfatase. Biochemistry 3:224-230.
15. **Rosner, J. L.** 1972. Formation, induction and curing of bacteriophage P1 lysogenes. Virology 48:679-689.
16. **Snyder, S. H., and E. D. Hendley.** 1971. Sensitive fluorometric and radiometric assays for monoamine oxidase and diamine oxidase, p. 741-746. *In* H. Tabor and C. W. Tabor (ed.), Methods in enzymology, vol. 17B. Academic Press Inc., New York.
17. **Zubay, G., D. E. Morse, W. J. Schrenk, and J. H. M. Miller.** 1972. Detection and isolation of the repressor protein for the tryptophan operon of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 69:1100-1103.