JOURNAL OF BACTERIOLOGY, Feb. 1981, p. 796-802
0021-9193/81/020996-07$02.00/0

Vol. 145, No. 2

Regulation of Derepressed Synthesis of Arylsulfatase by Tyramine Oxidase in *Salmonella typhimurium*

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The participation of tyramine oxidase in the regulation of arylsulfatase synthesis in *Salmonella typhimurium* was studied. Arylsulfatase synthesis was repressed by inorganic sulfate, cysteine, methionine, or taurine. This repression was relieved by tyramine, octopamine, or dopamine, which induced tyramine oxidase synthesis, although the level of arylsulfatase activity was very low. The induction of tyramine oxidase and derepression of arylsulfatase by tyramine were strongly inhibited by glucose and ammonium chloride, and the repression of both enzymes was relieved by use of xylose as a carbon source after consumption of glucose or by use of tyramine as the sole source of nitrogen, irrespective of the carbon source used. The initial rates of tyramine uptake by cells grown with glucose and xylose were similar. Results with tyramine oxidase-constitutive mutants showed that constitutive expression of the tyramine oxidase gene resulted in derepression of arylsulfatase synthesis in the absence of tyramine. Thus, catabolite and ammonium repressions of arylsulfatase synthesis and the induction of the enzyme by tyramine seem to reflect the levels of tyramine oxidase synthesis. These results in *S. typhimurium* support our previous finding that the specific regulation system of arylsulfatase synthesis by tyramine oxidase is conserved in enteric bacteria.

Arylsulfatase synthesis in *Klebsiella aerogenes* is repressed by a repressor molecule (*atsR*) that has been activated by a corepressor, such as inorganic sulfate, cysteine, or related compounds, and this repression is relieved by the addition of tyramine (1, 2). Recently, by using tyramine oxidase-deficient (*tynA*) and tyramine oxidase-constitutive (*tynP tynR*) mutants, we demonstrated that the expression of tyramine oxidase induced by tyramine results in derepression of the *atsA* gene specifying arylsulfatase, which is closely linked to *tynA* (9, 14, 16). Tyramine and catechol amines are oxidized by tyramine oxidase to hydroxyphenylacetoaldehyde compounds and ammonium ions, and the enzyme synthesis is subject to catabolite and ammonium repressions (9, 15, 18). Intergeneric hybrid strains, formed by transfer of the *ats* and *tyn* genes between *Escherichia coli*, *Salmonella typhimurium*, and *Citrobacter freundii* suggested that the system of regulation of arylsulfatase synthesis in enteric bacteria is conserved more than the structure (11, 12) or function of enzyme protein during evolution (21).

Recently, however, Henderson and Milazzo suggested that our model for regulation of arylsulfatase synthesis in enteric bacteria is not applicable to *S. typhimurium* LT2, since in this organism arylsulfatase synthesis was not subject to ammonium repression, and glucose might repress arylsulfatase synthesis by the exclusion of the inducer of the enzyme (7).

In the present study, we show that *S. typhimurium* LT2 synthesizes a very low level of arylsulfatase under the control of tyramine oxidase and that the apparent pattern of regulation of arylsulfatase synthesis by tyramine and carbon and nitrogen sources is due to its action on tyramine oxidase synthesis.

MATERIALS AND METHODS

Strains. *S. typhimurium* LT2 was used as the wild-type strain, and mutant strains were isolated from it.

Cultures and growth conditions. Bacteria were grown aerobically in a chemically defined medium composed of 0.1 M potassium phosphate buffer (pH 7.2); 0.01% $MgCl_2 \cdot 6H_2O$; 0.01% each of NaCl, $MnCl_2 \cdot 4H_2O$, $CaCl_2$, and $FeCl_3 \cdot 6H_2O$; and carbon, nitrogen, and sulfur compounds. Unless otherwise mentioned, 0.5% xylose and 0.1% NH_4Cl were used as carbon and nitrogen sources. The sulfur compounds used in each experiment are described below. Tyramine (3 mM) was used as a derepressing substrate for arylsulfatase synthesis or as an inducer for tyramine oxidase. The glucose-tyramine (GT) plates used for isolating the regulatory mutants of tyramine oxidase and arylsulfatase synthesis contained minimal medium with 1.5 mM tyramine as the sole source of nitrogen and 0.5% glucose as the carbon source. Growth was followed in a Klett-Summerson colimeter (590 to 660 nm).

Isolation of mutants. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and chloramphenicol was achieved as described by Sklar (20). The mutagenized cells were spread on GT plates at about

10^8 cells per plate and incubated at 30°C for 3 days. Colonies that grew on GT plates were picked and streaked to obtain single colonies. Wild-type cells (strain LT2) grew very slowly in GT medium, because their synthesis of tyramine oxidase, which is required for utilization of tyramine as the nitrogen source, is repressed by glucose.

Enzyme assay. Arylsulfatase activity was assayed as described previously (3). One unit of arylsulfatase activity was defined as the amount causing formation of one nanomole of *p*-nitrophenol per minute at 30°C. Tyramine oxidase activity was assayed by a radiometric procedure with [^{3}H]tyramine (15) or with an ammonium-selective electrode as described previously (10). One unit of tyramine oxidase activity was defined as the amount metabolizing one nanomole of tyramine per minute at 30°C.

Assay of tyramine uptake. Cells grown on xylose or glucose were harvested after three doublings, washed twice with 0.05 M phosphate buffer (pH 7.2) at 5°C, and suspended in the same volume of cold buffer solution. Uptake of radioactive tyramine was measured at 30°C in 1 ml of reaction mixture containing various amounts of [^{3}H]tyramine, 0.05 M phosphate buffer, 5 mM xylose or glucose, and 5 mg (dry weight) of cells. At intervals, cells were filtered through membrane filters (0.45-μm pore size; Toyo Roshi Co., Tokyo) and quickly washed three times with 3 ml of ice-cold 0.05 M phosphate buffer at room temperature. For measurements of the initial rate of uptake, a sample (0.2 ml) of cell suspension was rapidly pipetted into an equal volume of prewarmed medium containing an appropriate concentration of isotope. The initial rates of uptake were calculated from the linear uptake determined at 30-s intervals for 2 min. Reaction mixture at zero time of incubation was used as a control.

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

Preparation of enzyme extracts. Arylsulfatase was purified from *S. typhimurium* LT2 by a method similar to that used in purifying the enzyme from *K. aerogenes* W70 (17). The enzyme was purified about 250-fold and had a specific activity of 21 nmol/min per mg of protein. Tyramine oxidase was prepared from the membrane fraction of cells grown in xylose-NH_4Cl medium with tyramine as described previously (10).

Proteins were assayed by sodium deoxycholate trichloroacetic acid precipitation by the method of Lowry et al. as modified by Peterson (18).

RESULTS

Arylsulfatase and tyramine oxidase synthesis under various conditions. The levels of arylsulfatase and tyramine oxidase in cells of *S. typhimurium* LT2 were measured under various conditions. Table 1 shows the two enzyme activities in cells grown in synthetic media with xylose or glucose as the sole carbon source, with inorganic sulfate, cysteine, methionine, or taurine as the sole sulfur source, and with and without tyramine. Tyramine oxidase and arylsulfatase were synthesized only when cells were grown on xylose in the presence of tyramine. Arylsulfatase synthesis was repressed when cells were grown with inorganic sulfate, cysteine, methionine, or taurine in the absence of tyramine. The repressions by these sulfur compounds were relieved by addition of tyramine. A concentration of 3 mM tyramine was optimal for tyramine oxidase induction and for derepression of arylsulfatase, although 0.1 mM tyramine caused significant syntheses of these enzymes. Addition of dopamine or octopamine, but not norepinephrine, instead of tyramine to the same media caused the syntheses of both enzymes.

The syntheses of both enzymes were repressed when the cells were grown on glucose with ammonium chloride even in the presence of tyra-

TABLE 1. *Levels of arylsulfatase and tyramine oxidase in cells of S. typhimurium LT2 grown in various media*[a]

Supplement			Enzyme activity (U/mg of cells)	
Carbon source (0.5%)	Sulfur source (3 mM)	Tyramine (mM)	Arylsulfatase	Tyramine oxidase
Xylose	Na_2SO_4	—[b]	0.024	<0.01
Xylose	Na_2SO_4	0.1	0.122	0.75
Xylose	Na_2SO_4	1.0	0.241	0.83
Xylose	Na_2SO_4	3.0	0.382	0.99
Xylose	Na_2SO_4	5.0	0.263	0.70
Xylose	Na_2SO_4	10.0	0.142	0.65
Xylose	Cysteine	—	0.026	<0.01
Xylose	Cysteine	3.0	0.231	0.58
Xylose	Methionine	—	0.016	<0.01
Xylose	Methionine	3.0	0.382	1.08
Xylose	Taurine	—	0.006	<0.01
Xylose	Taurine	3.0	0.355	0.98
Glucose	Na_2SO_4	—	0.027	<0.01
Glucose	Na_2SO_4	3.0	0.029	0.03
Glucose	Cysteine	—	0.004	<0.01
Glucose	Cysteine	3.0	0.025	0.13
Glucose	Methionine	—	0.013	<0.01
Glucose	Methionine	3.0	0.016	<0.01
Glucose	Taurine	—	0.005	<0.01
Glucose	Taurine	3.0	0.031	<0.01
Glucose	Na_2SO_4	10.0[c]	0.335	0.93

[a] NH_4Cl was used as a nitrogen source except where indicated. When the turbidity became 20 Klett units, tyramine was added to the medium. Cells were harvested when the density reached about 160 Klett units.

[b] —, None.

[c] Tyramine (10 mM) was used as the sole source of nitrogen. Cells were cultured for 2 days, because growth was very slow.

mine. When *S. typhimurium* cells, like *K. aerogenes* (16), were grown in glucose-Na_2SO_4 medium with tyramine as the sole source of nitrogen, the syntheses of tyramine oxidase and arylsulfatase were relieved from glucose repression (Table 1).

The arylsulfatase activity of *S. typhimurium* LT2 under derepressing conditions with tyramine was about 10^3 times less than that of *K. aerogenes* W70. Thus, the K_m and V_{max} values of purified arylsulfatase of *S. typhimurium* LT2 were determined. The K_m and V_{max} values of *S. typhimurium* arylsulfatase (estimated from Lineweaver-Burk plots) were 1.3×10^{-2} M and 0.16 μmol/min per mg of protein, respectively, for *p*-nitrophenyl sulfate and 2.1×10^{-3} M and 0.07 μmol/min per mg of protein, respectively, for *p*-nitrocatechol sulfate. Thus, the K_m value for *p*-nitrophenyl sulfate is about 14 times that of *K. aerogenes* W70, and the V_{max} values for *p*-nitrophenyl sulfate and *p*-nitrocatechol sulfate are about 5.4×10^2 and 1.4×10^3 times less, respectively, than those of *K. aerogenes*. No activity was detected for indoxyl sulfate which has been used as an indicator of *K. aerogenes* arylsulfatase in agar plates in genetic analyses (2, 9).

We also measured the K_m and V_{max} values of membrane-bound tyramine oxidase of *S. typhimurium* LT2 prepared as described previously (10). Most of the enzyme was detected in the membrane fraction of the cells. The apparent K_m and V_{max} values for tyramine were determined as 3.8×10^{-4} M and 1.1 nmol/min per mg of protein, respectively, with [^{3}H]tyramine, and 4.0×10^{-4} M and 1.1 nmol/min per mg of protein, using an ammonium-selective electrode. The K_m and V_{max} values were 1.4×10^{-4} M and 0.4 nmol/min per mg of protein, respectively, for dopamine and 2.5×10^{-3} M and 1.8 nmol/min per mg of protein, respectively, for octopamine. No activity was observed with norepinephrine as reported previously (10). These K_m values were not significantly different from those of the enzyme of *K. aerogenes*, but the V_{max} values were about one-tenth to one half of those of the latter enzyme.

Catabolite repression of tyramine oxidase and arylsulfatase syntheses. The results in Table 1 suggest that the synthesis of tyramine oxidase is subject to catabolite repression by glucose, and this seems to result in failure of tyramine to derepress arylsulfatase synthesis. The effects of glucose starvation on the syntheses of tyramine oxidase and arylsulfatase in the presence of 0.5% xylose and 3 mM tyramine are shown in the differential plot (Fig. 1B). The lag period in the syntheses of tyramine

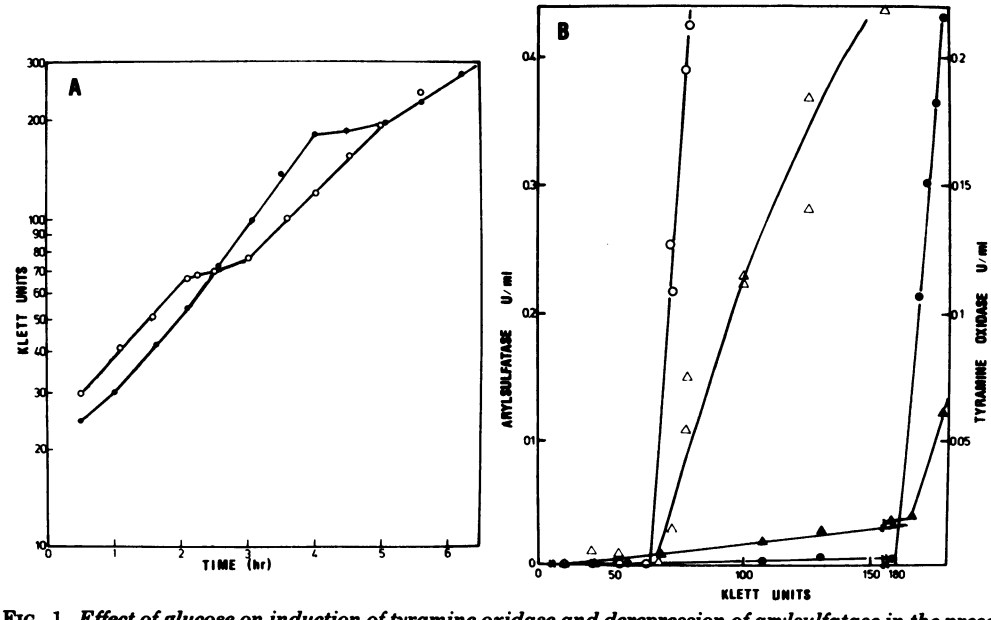

FIG. 1. *Effect of glucose on induction of tyramine oxidase and derepression of arylsulfatase in the presence of tyramine. Cells from an overnight culture of strain LT2 were washed with saline and suspended in medium containing 0.5% xylose, 0.1% NH_4Cl, and 3 mM tyramine with the indicated amounts of glucose. Symbols: (A) ○, growth with 0.02% glucose; ●, growth with 0.04% glucose; (B) ○, tyramine oxidase activity with 0.02% glucose; ●, tyramine oxidase activity with 0.04% glucose; △, arylsulfatase activity with 0.02% glucose; ▲, arylsulfatase activity with 0.04% glucose.*

oxidase and arylsulfatase increased as the concentration of glucose was increased. Syntheses of arylsulfatase and tyramine oxidase occurred only after consumption of the glucose. After the beginning of tyramine oxidase synthesis, the growth rate decreased from a doubling time of about 68 to 95 min, values which represent the rates with glucose and xylose, respectively, as carbon sources. Thus, glucose seems to repress tyramine oxidase synthesis, and this repression probably restricts derepression of arylsulfatase.

Another possible explanation of the effect of glucose is that it may inhibit tyramine transport into the cells when they are grown in glucose-NH_4Cl medium in the presence of tyramine as an inducer. To study this possibility, we measured the uptake of tyramine into cells grown on glucose or xylose (Fig. 2). No difference was found in the tyramine uptakes by cells grown with glucose and xylose. These results also suggest that tyramine entered the cells by passive diffusion, since tyramine uptake did not follow saturation kinetics. Glucose also did not interfere kinetically with the uptake of tyramine, since no difference was found in the tyramine uptakes in the presence of glucose and xylose, respectively.

Derepression of arylsulfatase synthesis by a tyramine oxidase-constitutive mutant without tyramine. To clarify the participation of tyramine oxidase in the derepression of arylsulfatase synthesis in *S. typhimurium* LT2, we isolated constitutive mutants of tyramine oxidase by the growth method described previously (14). Wild-type strain LT2 grew very slowly (doubling time, about 14 h) on minimal medium with tyramine as the sole source of nitrogen when glucose was used as the carbon source (GT medium). When strain LT2 was treated with *N*-nitro-*N'*-nitro-*N*-nitrosoguanidine and then plated on GT medium, mutants arose after 3 days at 30°C. Among 40 presumed regulatory mutants for synthesis of tyramine oxidase, four mutant strains, LTTC2, LTTC3, LTTC12, and LTTC15, apparently synthesized tyramine oxidase constitutively without tyramine as an inducer. When the mutant strains were grown with inorganic sulfate or methionine, arylsulfatase repression was relieved without tyramine (Table 2). The syntheses of tyramine oxidase and arylsulfatase in these mutants were still repressed by glucose, although they were partially insen-

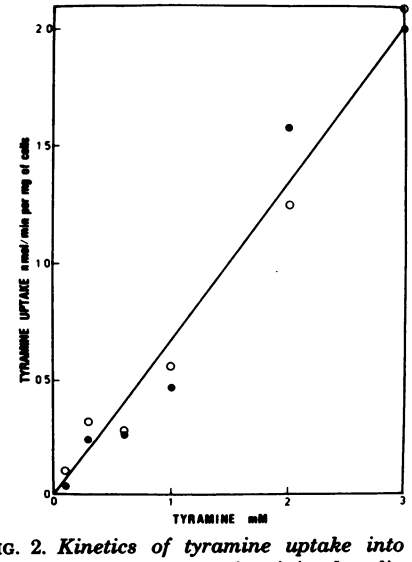

FIG. 2. *Kinetics of tyramine uptake into cells. Strain LT2 cells were grown in minimal medium with 0.5% glucose* (○) *or xylose* (●). *The amount of tyramine taken up at 30°C in 30 s was determined as described in the text.*

TABLE 2. *Levels of arylsulfatase and tyramine oxidase in mutant strains of S. typhimurium*[a]

Strain	Carbon source (0.5%)	Supplement sulfur source (3 mM)	Tyramine (3 mM)	Enzyme activity (U/mg of cells)	
				Arylsulfatase	Tyramine oxidase
LTTC2	Xylose	Na_2SO_4	−	0.199	0.64
	Xylose	Na_2SO_4	+	0.259	0.70
	Xylose	Methionine	−	0.209	0.36
	Xylose	Methionine	+	0.256	0.49
	Glucose	Na_2SO_4	−	0.020	0.02
	Glucose	Na_2SO_4	+	0.031	0.08
	Glucose	Methionine	−	0.017	0.06
	Glucose	Methionine	+	0.030	0.15
LTTC3	Xylose	Na_2SO_4	−	0.074	0.65
	Xylose	Na_2SO_4	+	0.086	0.69
	Xylose	Methionine	−	0.070	0.24
	Xylose	Methionine	+	0.086	0.54
	Glucose	Na_2SO_4	−	0.042	0.15
	Glucose	Na_2SO_4	+	0.058	0.34
	Glucose	Methionine	−	0.051	0.07
	Glucose	Methionine	+	0.073	0.23
LTTC12	Xylose	Na_2SO_4	−	0.140	0.47
	Xylose	Na_2SO_4	+	0.366	1.12
	Xylose	Methionine	−	0.156	0.88
	Xylose	Methionine	+	0.254	1.17
	Glucose	Na_2SO_4	−	0.121	0.22
	Glucose	Na_2SO_4	+	0.178	0.57
	Glucose	Methionine	−	0.121	0.19
	Glucose	Methionine	+	0.147	0.44
LTTC15	Xylose	Na_2SO_4	−	0.230	0.47
	Xylose	Na_2SO_4	+	0.692	1.27
	Xylose	Methionine	−	0.300	0.43
	Xylose	Methionine	+	0.163	0.75
	Glucose	Na_2SO_4	−	0.055	0.02
	Glucose	Na_2SO_4	+	0.068	0.04
	Glucose	Metionine	−	0.040	0.03
	Glucose	Methionine	+	0.065	0.06

[a] Cells were grown in minimal medium containing 0.1% NH_4Cl as a nitrogen source and harvested after approximately three doublings.

sitive to catabolite repression. These results show that tyramine was required for derepression of arylsulfatase only as an inducer of tyramine oxidase synthesis. The apparent repression by glucose is just an effect on tyramine oxidase; that is, synthesis of arylsulfatase itself is not directly subject to catabolite repression. The results clearly show that inhibition of enzyme synthesis by glucose is not due to "inducer exclusion."

Relief of ammonium repression. It is known that ammonium ions repress the syntheses of some nitrogen-dissimilating enzymes in *E. coli* (19), *S. typhimurium* (5), and *K. aerogenes* (5, 19) when the cells are grown in glucose medium, and that this repression is relieved when the cells are grown with a poor nitrogen source. Catabolite repression of tyramine oxidase by glucose in *K. aerogenes* was relieved when tyramine was used as a nitrogen source, even in the presence of glucose (15, 16). However, Henderson and Milazzo could not detect a repressive effect of any concentration of NH_4Cl on arylsulfatase synthesis in *S. typhimurium* (7). Thus, we carried out kinetic studies to see whether catabolite repression of tyramine oxidase and arylsulfatase in *S. typhimurium* is reversed when tyramine is used as a nitrogen source in the presence of glucose. Cells of the tyramine oxidase-constitutive strain LTTC2 were grown in chemically defined medium with 10 mM tyramine and 0.5% glucose, with ammonium chloride at two different concentrations (Fig. 3). The lag periods before the syntheses of tyramine oxidase and arylsulfatase increased progressively with increase in the concentration of ammonium salts added. Both enzymes were synthesized when the ammonium ions had been consumed during incubation. After the beginning of enzyme synthesis, the growth rate decreased to about a quarter of that in the initial stage of growth (doubling time, 65 min), which was the same as that of strain LTTC2 with tyramine as the sole nitrogen source (doubling time, 240 min). Thus, induction of tyramine oxidase synthesis in *S. typhimurium* escaped from catabolite repression by glucose, and this resulted in derepression of arylsulfatase synthesis, when tyramine was used as the sole nitrogen source in the presence of glucose.

The results with tyramine oxidase-constitutive mutants show that the synthesis of tyramine oxidase is essential for derepression of arylsulfatase synthesis.

DISCUSSION

Arylsulfatase has been found in some strains of the family *Enterobacteriaceae*, such as *Klebsiella*, *Enterobacter* (1, 21), *Salmonella* (6, 7), *Serratia* (13, 21), and *Proteus* (4, 8, 21). How-

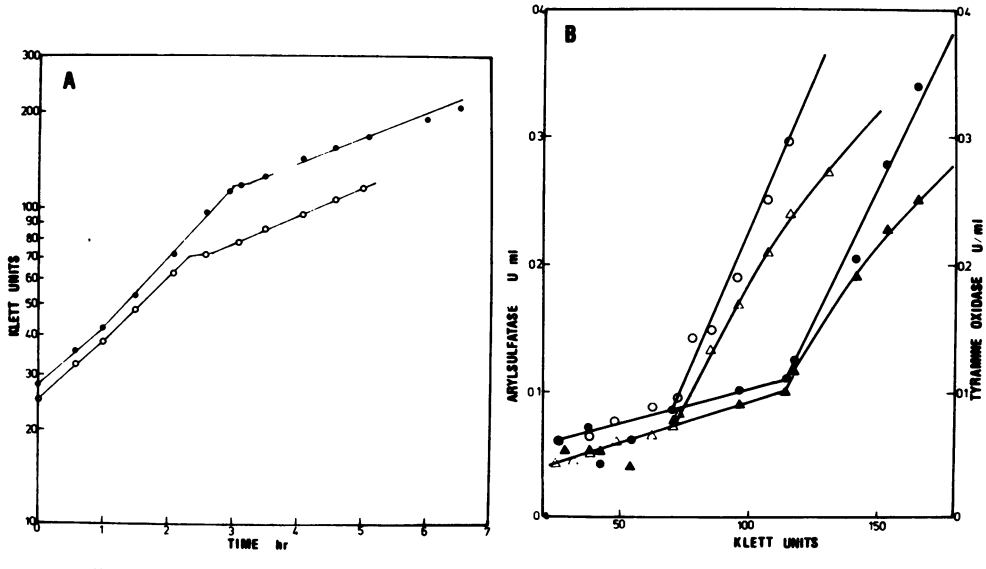

FIG. 3. *Effect of ammonium chloride on the induction of tyramine oxidase and derepression of arylsulfatase in the presence of glucose. Cells from an overnight culture of strain LTTC2 were washed with saline and suspended in medium containing 0.5% glucose and 10 mM tyramine with the indicated amounts of NH_4Cl. Symbols: (A) ○, growth curve with 0.01% NH_4Cl; ●, 0.02% NH_4Cl; (B) ○, tyramine oxidase activity in medium initially containing 0.01% NH_4Cl; ●, 0.02% NH_4Cl. Arylsulfatase activity in medium initially containing 0.01% NH_4Cl (△) and 0.02% NH_4Cl (▲).*

ever, the levels of activity in these strains varied considerably, possibly owing to differences in enzyme structure or in the mechanisms of regulation of enzyme synthesis. To elucidate the genetic regulation of arylsulfatase synthesis, we chose *K. aerogenes* W70, since *E. coli* K-12 and *S. typhimurium* LT2, the bacterial strains most widely used in genetic analyses, produce little or no arylsulfatase under normal conditions. On the basis of genetic studies using the general transducing phage PW52 and introducing the coliphage P1 or F′ episomes into *K. aerogenes*, we proposed a model of genetic regulation of arylsulfatase synthesis (9). Furthermore, in immunological studies we showed that there is a similar regulation system of arylsulfatase synthesis in enteric bacteria (12, 21) and in intergeneric hybrid strains between *K. aerogenes* and *E. coli* or *S. typhimurium* (11).

In this paper, we show that arylsulfatase from *S. typhimurium* LT2 differs greatly from the enzyme of *K. aerogenes* (17) and *Serratia marcescens* (13) in its K_m and V_{max} values. Comparative studies on the regulation of the syntheses of tyramine oxidase and arylsulfatase and genetic studies on mutant strains, such as *tynA*, *tynP*, or *tynP tynR*, in *K. aerogenes* led us to the interesting conclusion that the apparent level of derepressed synthesis of arylsulfatase by tyramine under conditions of catabolite and ammonium repression is just a reflection of the regulation pattern of tyramine oxidase synthesis (9, 14, 16). The experiments reported here clearly show that the regulation of arylsulfatase synthesis in *S. typhimurium* is similar to that in *K. aerogenes*, except for the effect of methionine, although this idea has been strongly challenged by others (7). Arylsulfatase synthesis in *K. aerogenes* is repressed by inorganic sulfate or cysteine, but not by methionine or taurine as the sole source of sulfur (2), and this nonrepressed synthesis of arylsulfatase is not subject to catabolite repression (3). However, methionine and taurine repressed arylsulfatase synthesis in *S. typhimurium*, as in *S. marcescens* (13).

We also showed that the initial rates of tyramine uptake in *S. typhimurium* cells, as in *K. aerogenes* (3), did not differ in cells grown with glucose and xylose, irrespective of whether carbohydrate was present in the reaction mixture. Since an efflux of free tyramine from the cells into the medium may occur rapidly, the uptake of tyramine as a carbon source with glucose into growing cells reported by others (7) may not represent the true incorporation rate of tyramine into the cells. We also ruled out the possibility that the repression of arylsulfatase synthesis by glucose was due to exclusion of the inducer by glucose, as suggested by Henderson and Milazzo (7), since mutant cells forming tyramine oxidase constitutively were sensitive to catabolite repression by glucose in the absence of tyramine. Furthermore, we demonstrated ammonium repression of the synthesis of tyramine oxidase in wild-type and tyramine oxidase-constitutive strains in *S. typhimurium*. The reason that other workers could detect no influence of NH_4Cl on arylsulfatase synthesis in *S. typhimurium* was probably that arylsulfatase activity was measured in cells grown on tyramine as the sole source of carbon or nitrogen or both, since they reported that the maximal level of enzyme was attained after consumption of glucose (7). Ammonium repression is observed in the presence of glucose, as reported first by Prival and Magasanik in their article on regulation of the syntheses of histidase and proline oxidase (19).

Thus, the present paper and the results on *S. marcescens* arylsulfatase (13) support our previous finding that the specific regulation system of arylsulfatase synthesis by tyramine oxidase is conserved better than is the structure or function of the enzyme protein in the family *Enterobacteriaceae* (11), although different species show differences in the degree of catabolite repression and in susceptibility to methionine.

ACKNOWLEDGMENTS

We thank H. Mitani for preparing purified arylsulfatase and M. Oka and Y. Matsumoto for valuable discussions.

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