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Thiolutin-Resistant Mutants of *Salmonella typhimurium*

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Spontaneous mutants of *Salmonella typhimurium* isolated in our laboratory from thiolutin-containing tryptone agar plates are partially resistant to thiolutin in enriched media. In minimal media, they are not resistant. The mutants are not temperature sensitive but fail to support the development of phage P22 at higher temperatures (40°C). Thiolutin did not interfere with RNA polymerase or nucleotide kinase in in vitro experiments. However, thiolutin did inhibit the rate of incorporation of exogenous uridine into the cellular pool and consequently the acid-precipitable material. It appears that one site of action of thiolutin is at the membrane level.

Thiolutin (2), a broad-spectrum sulfur-containing antibiotic, is reported to inhibit mRNA chain elongation (7). Thiolutin also inhibits RNA synthesis in yeast cells, and the effect is reversible (5, 16). Although Sivasubramanian and Jayaraman suggested that thiolutin interferes with the initiation of RNA synthesis in *Escherichia coli*, inhibition of RNA synthesis by thiolutin was not demonstrated in vitro (12). Subsequently, Sivasubramanian and Jayaraman (13) showed that thiolutin-resistant mutants of *E. coli* map at two different loci. It has been reported from this laboratory that spontaneous thiolutin-resistant mutants of *Salmonella typhimurium* are unable to support the development of phage P22 at higher temperatures (40°C), although the mutants themselves are not temperature sensitive (6). This report deals with the isolation and properties of these mutants and the mode of action of thiolutin.

MATERIALS AND METHODS

Bacterial strains. *S. typhimurium* wild-type strain 18 and its histidine requiring mutant (strain 153) were obtained from M. Levine of the University of Michigan, Ann Arbor, Mich.

Chemicals. Thiolutin was a gift from N. Belcher, Pfizer Inc., New York, N.Y. Rifampin was purchased from Sigma Chemical Co., St. Louis, Mo. [^{3}H]uridine (5,200 Ci/mol) was obtained from Bhabha Atomic Research Centre, Bombay, India. [^{3}H]UTP (35 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. [^{3}H]thymidine (6,700 Ci/mol) was purchased from New England Nuclear Corp. 2,5-Diphenyloxazole (PPO) and 1,4-(2,4-dimethyl-5-phenyloxazolyl)benzene, (dimethyl-POPOP) were the products of Amersham Corp., Arlington Heights, Ill. Nitrocellulose membrane filters (0.45 μm) were from Schleicher & Schuell, Inc., Keene, N.H. Other chemicals were also commercial preparations of analytical grade.

Growth medium. The cells were grown in minimal medium (MM), Luria broth (LB), or Casamino Acid-supplemented MM (M9CAA). The compositions of the media have been described elsewhere (3, 8, 14). In case of histidine-requiring strains, MM was supplemented with histidine (20 μg/ml).

Measurement of growth rate. Growth rate was measured by following the optical density at 610 nm of the cell suspension in a Hilger colorimeter. An absorbancy of 0.2 of a cell suspension growing in MM and LB represents 2.6×10^8 and 1.2×10^8 cells per ml, respectively.

Determination of burst size. Exponentially growing cells were infected with phage at a multiplicity of infection of 10. After we allowed 5 min for adsorption, the unadsorbed phage were neutralized by treatment for 5 min with antiserum (final K = 2). Infective centers were determined by plating the antiserum-treated cell suspension, after suitable dilution, on indicator strain 18 unless otherwise stated. Properly diluted samples of the infected cells were incubated at the requisite temperature. To determine the phage yield, the lysate was suitably diluted and plated on tryptone agar plates with the susceptible strain as the plating bacteria. The burst size was calculated from the number of infective centers irrespective of the number of cells used for infection.

Measurement of the rates of incorporation of [^{3}H]uridine and [^{3}H]thymidine into trichloroacetic acid-insoluble fraction. The cells were grown in M9CAA from an inoculum grown overnight. When the cell suspension reached a density of 2.6×10^8 cells per ml, the infection was carried out with the phage at the desired multiplicity of infection. At different times after infection, samples (0.5 ml) were pulsed for 1 min with [^{3}H]uridine (1 nmol containing 2×10^5 cpm or [^{3}H]thymidine (2 nmole, containing 10^6 cpm) at the desired temperature with constant shaking. The reaction was stopped by the addition of equal volume of cold 10%

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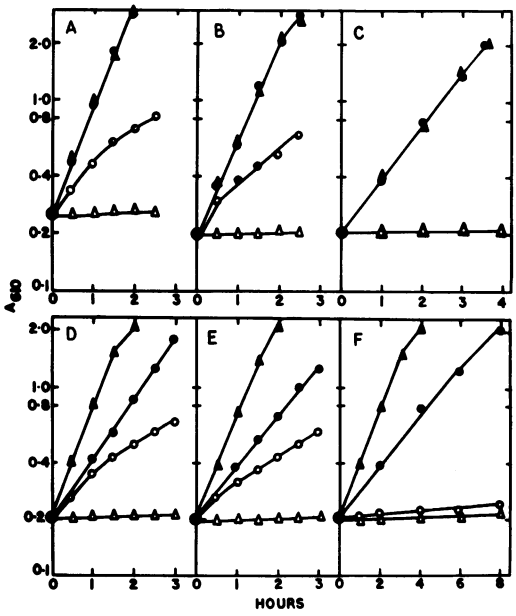

FIG. 1. Growth rates of the mutants in different media in the presence and absence of thiolutin. The cells were grown in either LB (A and D), M9CAA (B and E), or MM (C and F) at 37°C. When the growing cell suspension reached a cell density of approximately 2.6×10^8 cells per ml, thiolutin was added at a concentration of 8 μg/ml. Panels A, B, and C represent growth rates of 18/MC4. The growth rate of strain 18 was followed as control. Panels D, E, and F represent growth rates of the thiolutin-resistant mutant 153/MC4. Other conditions are as described above, except that the MM contained histidine at a final concentration of 20 μg/ml, since strain 153 is a histidine-requiring strain. The growth of 153 was followed as a control. Symbols: ▲, control cells in the absence of thiolutin; △, control cells in the presence of thiolutin; ●, mutant in absence of thiolutin; and ○, mutant in presence of thiolutin.

trichloroacetic acid. The tubes were placed in an ice bath for 30 min before the contents were filtered and washed with 20 ml of ice-cold 5% trichloroacetic acid. The filters were dried and counted in a Mark II liquid scintillation counter, of Nuclear-Chicago Corp., Des Plaines, Ill., using a scintillation solution which contained 6 g of PPO and 1 g of dimethyl-POPOP per liter of toluene. Corrections were made in all cases for the corresponding time zero blanks.

Measurement of the rate of incorporation of [^{3}H]uridine into cellular pool. The rate of incorporation of [^{3}H]uridine into cellular pool was calculated by substracting the amount of radioactivity incorporated into the trichloroacetic acid-insoluble fraction from the total amount incorporated by the cell (11). This method is justifiable because of the large pool size of nucleotides in *S. typhimurium* (11).

Assay of RNA polymerase. Cells were grown up to early log phase in nutrient broth containing glucose, harvested, and then disrupted in Aminco French pressure cell (18,000 lb/in^2). The extract was centrifuged at 20,000 × *g* for 5 min. The supernatant was centrifuged at 100,000 × *g* for 15 min. The S100 (16 to 20 mg of protein per ml) was tested for polymerase activity. The assay was performed by the method of Burgess (1), using calf thymus DNA as template and by the method of Harshey and Ramakrishnan (4).

Protein assay. The protein concentration in the crude extract was measured by the method of Lowry et al. (9).

Assay of thymidine kinase and uridine kinase. Exponentially growing cells of *S. typhimurium* were harvested and washed with 0.01 M Tris-hydrochloride (pH 7.8). Cells were ground with alumina at 4°C and suspended in 0.02 M Tris-hydrochloride buffer (pH 7.8). The suspension was centrifuged at 10,000 rpm for 20 min in Sorvall RC5B rotor SS34 at 4°C for removal of alumina and cell debris. The supernatant was used as the source of enzyme.

The enzyme activities were assayed by following the conversion of labeled thymidine and uridine to the respective monophosphates (10). For thymidine kinase assay, both ATP and GTP were used as phosphate donor, since the enzyme can utilize GTP and ATP.

RESULTS

Isolation of mutants. The thiolutin-resistant mutants were isolated as spontaneous mutants by plating about 10^9 cells on tryptone agar containing thiolutin (8 μg/ml). The resistant colonies were purified by streaking on thiolutin containing plates. The strains serially numbered from 153/MC2 to 153/MC5 were isolated from strain 153, whereas the strains serially marked from 18/MC2 to 18/MC9 were from strain 18. The frequency of spontaneous mutation was about 3×10^{-7}. The frequency of back mutation could not be determined due to the lack of a convenient selection procedure.

Growth rates of thiolutin-resistant mutants in different media in the presence and absense of thiolutin. Of the thiolutin-resistant mutants, 18/MC4 and 153/MC4 were the most defective in their ability to support phage developments. Hence, the properties of 18/MC4 and 153/MC4, the latter being slow growing (6), were studied in detail. Comparison of the growth rates of the parent strain and thiolutin-resistant mutants, in different media and in the presence and absence of thiolutin, clearly indicated that thiolutin resistance was dependent on the medium (Fig. 1). In enriched media, these mutants were partially resistant, whereas in MM they were susceptible. In MM, they behaved as conditional auxotrophs in the presence of thiolutin. Such dependency on growth medium has been reported for a rifampin-resistant mutant of *E. coli* (15). However, the addition of NaCl to the medium makes those mutants of *E. coli* resistant even in MM. For the thiolutin-resistant mutants of *S.*

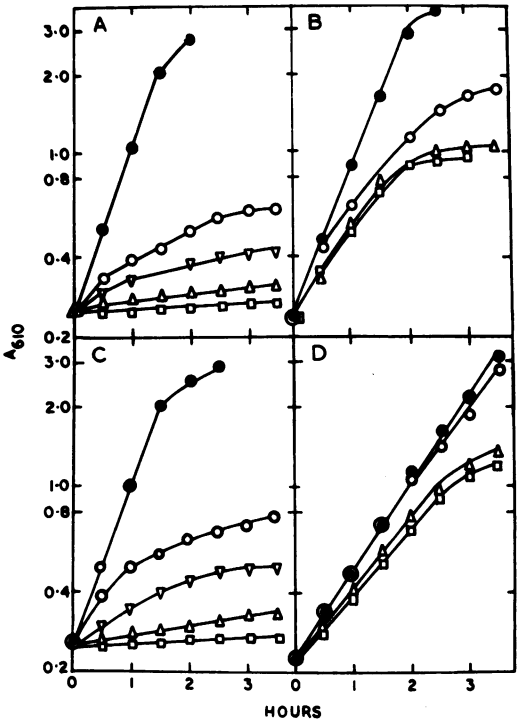

FIG. 2. Effect of thiolutin concentration on the growth rates of thiolutin-susceptible and -resistant strains of *S. typhimurium*. Growth rates of the strains 18 and 18/MC4 are presented in panels A and B, respectively, whereas panels C and D represent growth rates of 153 and 153/MC4. The cells were grown in LB. Symbols (concentration of thiolutin in micrograms per milliliter): ●, 0; ○,1; ▽, 2; △, 3; and □, 5.

typhimurium in this study, no such effect of NaCl was observed.

Effect of thiolutin concentration on the growth rates of the thiolutin-resistant strains. The effect of thiolutin concentration on the growth rates of the wild-type strains and two resistant strains was studied. The results indicate that the minimum inhibitory concentration of thiolutin for the wild-type (susceptible) strain is 3 μg/ml (Fig. 2). At a concentration of 1 μg/ml, the growth rate was 60% inhibited. The mutants appear to be partially resistant to thiolutin. Strain 153/MC4 is more resistant than is strain 18/MC4.

Effect of thiolutin on the rates of incorporation of uridine and thymidine into acid-precipitable material. Since thiolutin reportedly interferes with RNA synthesis in procaryotes (7, 12), mutants were tested for their ability to synthesize RNA in presence of thiolutin. The rate of DNA synthesis was also measured. The rates of both RNA and DNA synthesis, as measured by the rates of incorporation of precursors into macromolecules, were inhibited in presence of thiolutin (Fig. 3). The rate of DNA synthesis in the mutants was less inhibited than RNA synthesis. The rate of RNA synthesis was less inhibited in 153/MC4 than in 18/MC4.

Effect of thiolutin on RNA polymerase in vitro. The RNA polymerase activity present in the extracts of both wild-type and mutant strain of *S. typhimurium* was not inhibited by thiolutin (Table 1). Thiolutin, even at a concentration of 100 μg/ml, had no effect on RNA synthesis. Since calf thymus DNA was used as a template, the possibility of inhibition of promoter specific initiation of transcription could not be ruled out by this experiment.

Effect of thiolutin on nucleoside kinase activity. Thymidine kinase and uridine kinase activities in the cell-free extract of *S. typhimurium* were tested in the presence and absence of thiolutin. Thiolutin did not interfere with uridine and thymidine kinase activity (Tables 2 and 3).

Effect of thiolutin on the rate of transport of exogenous uridine into soluble pool. To determine whether the observed inhibition in the rate of RNA synthesis in vivo was caused by a reduced rate of transport of exogenous uridine, the rate of incorporation of exogenous uridine into intracellular pool and macromolecules was followed. To ascertain whether the inhibition of the rate of incorporation into the pool, if any, was the effect of inhibition of the rate of RNA synthesis, a similar experiment was carried out in the presence of rifampin, which is known to inhibit RNA synthesis. In presence of rifampin, the rate of RNA synthesis was decreased, and the pool of

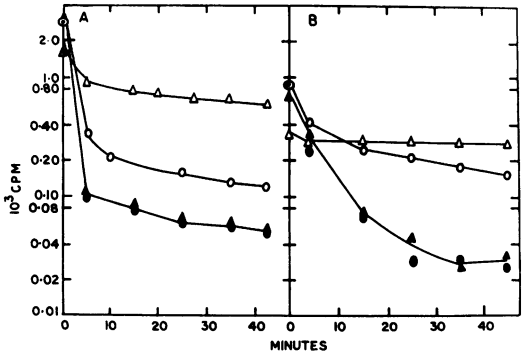

FIG. 3. Rates of RNA (A) and DNA (B) synthesis at 40°C in thiolutin-resistant mutants in the presence of thiolutin. Thiolutin-resistant mutants 18/MC4 and 153/MC4 and respective parent strains 18 and 153 were grown at 40°C in M9CAA. Thiolutin (5 μg/ml) was added to the exponentially growing cells (2.6×10^8 cells per ml). At different times after the addition of thiolutin, the rates of RNA and DNA synthesis were determined as described in Materials and Methods. Symbols: ●, 18; ○, 18/MC4; ▲, 153; and △, 153/MC4.

TABLE 1. Effect of thiolutin on RNA polymerase activity of *S. typhimurium* in vitro

Expt no.	Enzyme source	Radioactivity incorporated into RNA (cpm)		
		Without thiolutin	With thiolutin (μg/ml)	
			10	100
Expt 1[a]	18	1,058	797	1,147
	18/MC4	1,087	1,081	1,165
	153/MC4	1,331	947	1,188
Expt 2[b]	18	2,378		2,612
	18/MC4	4,234		3,338
	153/MC4	2,725		2,612

[a] Input radioactivity was 10,000 cpm per assay; without enzyme, 167 cpm.
[b] Input radioactivity was 40,000 cpm per assay; without enzyme, 403 cpm.

intracellular uridine remained unaffected. On the contrary, the rates of incorporation of exogenous uridine into the intracellular pool and into the macromolecules decreased in the presence of thiolutin (Fig. 4).

Growth of P22 in thiolutin-resistant mutants. The mutants were unable to support the development of phage P22 at higher temperature, i.e., 40°C (Table 4). The thiolutin-resistant mutants are, however, not temperature sensitive. Phage development was normal at low temperature (30°C) in the presence of thiolutin. The inability of these mutants to support phage development at higher temperature is independent of the growth media (data not presented). That such a defect in phage development is related to the thiolutin resistance is probable because these thiolutin-resistant mutants are independent spontaneous mutants.

Kinetics of phage P22-induced lysozyme synthesis in thiolutin-resistant strains in the presence of thiolutin. Although the number of productive phage particles produced in 153/MC4 and 18/MC4 is less at nonpermissive temperatures (40°C), the rates of DNA and lysozyme synthesis were comparable to those of the parent strain (6). When strains were infected with phage at 40°C in the presence of thiolutin, lysozyme was not induced in 18/MC4 but there was normal induction in 153/MC4 (Fig. 5). Although lysozyme was induced in 153/MC4 at 40°C, the cells did not lyse. Lysis could, however, be induced by the addition of chloroform. Lysozyme induction was studied in LB, so it was of interest to determine whether the situation was different in other media (Table 5). For 153/MC4, lysozyme was synthesized in enriched media (LB and M9CAA) at permissive and nonpermissive temperature and in the presence and absence of thiolutin. In MM, however, there was no enzyme induction in the presence of thiolutin, although synthesis took place when thiolutin was not present. A somewhat different situation

TABLE 2. Effect of thiolutin on thymidine kinase activity of *S. typhimurium*[a]

Incubation no.	Extract (μg of protein)	Thiolutin	Picomoles of [^{3}H]thymidine monophosphate formed per minute
1	80	−	13.5
2	80	+	14.05
3	160	−	29.5
4	160	+	24.5

[a] In incubation, the mixture contained (in a total volume of 0.1 ml) 52.5 mM Tris-hydrochloride (pH 7.8), 7.5 mM ATP, 7.5 mM $MgCl_2$, 40 μg of bovine serum albumin, 15 mM NaF, 1.3 nmol of [^{3}H]thymidine containing 5×10^5 cpm, 0.8 μg of thiolutin (wherever needed), and enzyme as indicated in the table. The rest of the procedure was as described by Ming et al. (10).

TABLE 3. Effect of thiolutin on uridine kinase activity of *S. typhimurium*[a]

Incubation no.	Extract (μg of protein)	Thiolutin (8 μg/ml)	Picomoles of [^{3}H]uridine monophosphate formed per minute
1	80	−	28.8
2	80	+	20.5
3	160	−	67.5
4	160	+	58.6
5	80	−	41.9
6	80	+	36.2
7	160	−	83.0
8	160	+	81.5

[a] For the assay of uridine kinase activity, the incubation mixture was as described in Table 2, footnote *a*, except that 1 nmol of [^{3}H]uridine containing 3×10^5 cpm was used. GTP (7.5 mM) was used as the phosphate donor in incubation numbers 1, 2, 3, and 4, whereas ATP was used as the phosphate donor in incubation numbers 5, 6, 7, and 8.

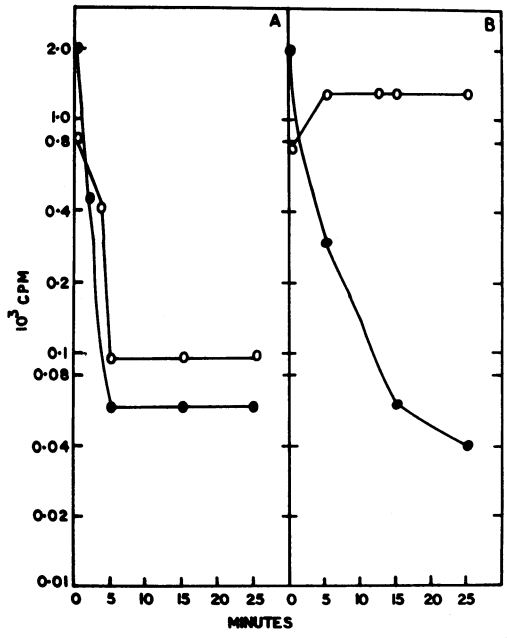

FIG. 4. Rates of incorporation of exogenous uridine into cellular pool and macromolecules in the presence of thiolutin (A) and rifampin (B). *S. typhimurium* strain 18, a thiolutin- and rifampin-susceptible strain, was grown in M9CAA. Exponentially growing cells (2.6×10^8 cells per ml) were divided into two batches. One batch (A) was treated with thiolutin (5 μg/ml) and the other batch (B) was treated with rifampin (100 μg/ml). The flasks were covered with black paper to protect the contents from light. At different times after the addition of thiolutin and rifampin, samples (0.5 ml) were taken, and the total amount of radioactivity incorporated into the cell and that into the macromolecules was measured as described in Materials and Methods. Symbols: ●, radioactivity in the trichloroacetic acid-precipitable fraction; ○, radioactivity in the cellular pool (small molecules).

was observed for 18/MC4. There was no induction of lysozyme at 40°C in the presence of thiolutin in any of these media tested. The enzyme was synthesized at permissive temperatures in the presence and absence of thiolutin in enriched media. In MM, there was no enzyme induction in the presence of thiolutin.

DISCUSSION

Thiolutin was reported to be an inhibitor of RNA polymerase (16). Khachatourians and Tipper (7) suggested that thiolutin inhibits RNA chain elongation in *E. coli*. Subsequently, Sivasubramanian and Jayaraman (12) indicated that thiolutin might interfere with the initiation of RNA synthesis. They could not demonstrate the inhibition of RNA synthesis in vitro, but the

TABLE 4. Infective particles produced in different thiolutin-resistant mutants at 30 and 40°C in the presence and absence of thiolutin[a]

Strain	Burst sizes at			
	30°C		40°C	
	−[b]	+[b]	−	+
18	440	35	440	0.4
18/MC2	390	330	30	22
18/MC3	354	370	38	14
18/MC4	20	20	7	7
18/MC5	364	390	20	20
18/MC6	330	400	15	37
18/MC7	440	340	36	25
18/MC8	340	400	30	40
18/MC9	360	400	30	72
153	280	32	300	0.5
153/MC2	260	230	10	30
153/MC3	330	400	40	18
153/MC4	52	48	0.9	0.2
153/MC5	250	280	22	25

[a] The experiment was carried out in LB. Similar results were obtained in M9CAA. In MM, burst sizes at 30 and 40°C in the absence of thiolutin were very similar to those obtained with cells growing in LB or M9CAA, but extremely low in the presence of thiolutin.

[b] − and +, Absence and presence of thiolutin, respectively.

suggestion was made from the inhibition of incorporation of precursor of RNA into macromolecules in vivo. However, they mentioned, as a personal communication with D. J. Tipper, that thiolutin may interfere with the nucleoside kinase activities. With calf thymus DNA as a template, the RNA polymerase activity of *S. typhimurium* could not be inhibited by thiolutin. However, thiolutin inhibits the incorporation of exogenous uridine and thymidine into macromolecules in vivo. Such inhibition was not observed in case of the thiolutin-resistant mutant 153/MC4, whereas the inhibition was partial in the mutant 18/MC4 (Fig. 3). In presence of thiolutin, the wild-type strain did not incorporate exogenous uridine into soluble pools, whereas in presence of rifampin, an inhibitor of RNA synthesis, incorporation of exogenous uridine into soluble pools remained unaffected (Fig. 4). Furthermore, thiolutin did not inhibit thymidine or uridine kinase activity (Tables 2 and 3). From these observations, it appeared that the mode of action of thiolutin might be either of the following: (i) thiolutin interferes with promoter-specific initiation of transcription, and (ii) thiolutin inhibits the cellular transport process. In the presence of thiolutin, exogenous uridine is not incorporated by the cells, and the effect is not the same as that of rifampin, an inhibitor of initiation of transcription. Therefore, we believe that one mode of action of thiolutin is the

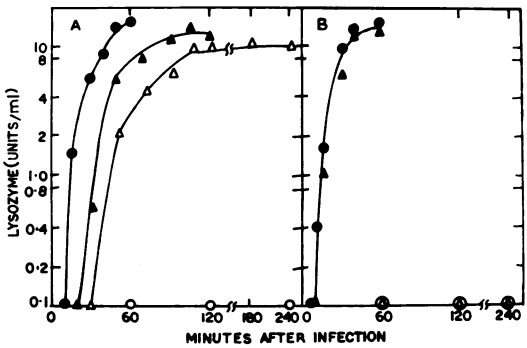

FIG. 5. Kinetics of lysozyme induction in strains 153/MC4 (A) and 18/MC4 (B) in presence of thiolutin after infection with P22 (C_1) at nonpermissive temperature. Thiolutin-resistant strains 153/MC4 and 18/MC4 and the respective parent strains (153 and 18) were grown in LB at 40°C. Exponentially growing cells at a density of 2.5×10^8 cells per ml were divided into two batches. To one batch, thiolutin (final concentration, 5 μg/ml) was added before the addition of the phage. Infection was carried out at a multiplicity of infection of 10. Collection of samples and assay of lysozyme were done as described in Materials and Methods. Symbols: ●, 153 or 18 in the absence of thiolutin; ○, 153 or 18 in the presence of thiolutin, ▲, 153/MC4 or 18/MC4 in the absence of thiolutin; and △, 153/MC4 or 18/MC4 in the presence of thiolutin.

inhibition of cellular transport processes. It is quite possible that there is more than one locus for thiolutin resistance, as reported for *E. coli* (13). Sivasubramanian and Jayaraman (13) have reported that there are two sites on *E. coli* genome, each of which confers partial resistance to thiolutin. Double mutants are resistant in minimal medium. Our mutants, being spontaneous mutants, most probably contain single mutation and hence are partially resistant. The mutant 153/MC4 seems to be more resistant than is 18/MC4.

The most interesting observation made with the thiolutin-resistant mutants was that these mutants preferentially interfere with the morphogenesis of phage P22 at 40°C. Noninfectious phage particles were produced at this temperature, although most of the phage functions, including lysozyme synthesis, were not affected (6). Although both mutants interfere with phage morphogenesis (6), they block phage development at different stages (6a), suggesting that these two mutants are not mutated at the same site. This was further confirmed from the studies on lysozyme induction in presence of thiolutin. The two mutants behaved differently when lysozyme synthesis was followed after phage infection in the presence of thiolutin. In enriched media at 30°C, both strains produced lysozyme in the presence of thiolutin. Lysozyme production is not much affected in 153/MC4 in either the presence or absence of thiolutin. In 18/MC4, however, thiolutin has no further effect on phage production at 40°C, yet at 40°C, lysozyme synthesis is inhibited in presence of thiolutin. It is quite possible that phage gene expression is more sensitive to thiolutin than is the host gene expression. Thus, thiolutin may also interfere with promotor-specific transcription. 153/MC4 shows no effect of thiolutin on either phage production or lysozyme synthesis. This may be due to impermeability of the cells toward thiolutin.

153/MC4 appears to be a permeability mutant, since thiolutin has no effect on lysozyme synthesis. Even when lysozyme is synthesized normally, no lysis of the cells takes place, possibly because of an alteration in the cell membrane. No explanation can be offered regarding the biochemical lesion in 18/MC4.

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TABLE 5. Effect of thiolutin on lysozyme synthesis in phage P22-infected strains, 18/MC4 and 153/MC4 growing in different media at permissive and nonpermissive temperatures

Strain	Lysozyme U/ml											
	LB				M9CAA				MM			
	30°C		40°C		30°C		40°C		30°C		40°C	
	−[a]	+[a]	−	+	−	+	−	+	−	+	−	+
18	11.0	0.9	10.5	0.8	9.0	1.0	10.0	1.0	13.0	0.0	12.0	0
18/MC4	9.5	6.5	11.0	0.5	7.0	3.5	8.0	0.5	10.0	1.0	11.0	0
153	10.5	0.5	10.5	0.25	13.0	0.25	12.0	0.25	12.0	0.25	12.5	0
153/MC4	9.0	8.5	9.0	9.0	9.0	7.0	10.5	8.0	11.0	1.0	11.5	1.0

[a] + and −, Presence and absence, respectively, of thiolutin. Wherever used, the final concentration of thiolutin was 5 μg/ml.

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