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Penicillin-Resistant Temperature-Sensitive Mutants of *Escherichia coli* Which Synthesize Hypo- or Hyper-Cross-Linked Peptidoglycan

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A group of *Escherichia coli* mutants which are ampicillin resistant at 32 C and which either are unable to grow or lyse at 42 C has been selected. These mutants have been classified by a number of characteristics: total peptidoglycan synthesis measured by [^{14}C]diaminopimelic acid incorporation, extent of cross-linking of the peptidoglycan which is synthesized, growth characteristics at the two temperatures, and morphology. Two especially interesting groups of mutants have been described. In one of these, a hypo-cross-linked peptidoglycan was synthesized at the nonpermissive temperature. Most of these organisms lysed at 42 C. In another group, the peptidoglycan synthesized at 42 C was hyper-cross-linked. Many of these organisms were spherical. Studies of revertants indicated that ampicillin resistance, temperature sensitivity, cross-linking, growth characteristics, and morphological changes may be related to a single mutational event in both of these groups.

The lethal effect of penicillins on *Escherichia coli* is thought to be due to the inhibition by these antibiotics of enzymes involved in the terminal stage of cell wall synthesis, the cross-linking of peptidoglycan strands in the cell wall (22). Three enzymatic activities have been identified which are inhibited by penicillins: the transpeptidase which catalyzes the cross-linking reaction (3, 11, 12), the D-alanine carboxypeptidase whose precise function is unknown but which might participate in reactions which limit the extent of cross-linking in peptidogylcan strands (13), and an endopeptidase (cross-link splitting enzyme) which could similarly be involved in either determining the final extent of cross-linking in the peptidoglycan or in some manner involved in the separation of cells or in the growth of the peptidoglycan (6, 8). It is also possible that several of these activities are catalyzed by one enzyme (11, 21).

One approach to the questions of the number of enzymes required to catalyze these activities and to their functions is to isolate mutants in which the various enzymes are altered. In addition, the isolation of a penicillin-resistant strain containing a penicillin-resistant enzyme would provide further evidence that that enzyme is, in fact, a killing site for penicillin. This approach is complicated by the possibility that more than one killing site for penicillin may exist (5, 8, 9, 23, 24). Many studies of penicillin-resistant mutants in the past have failed to result in the isolation of strains in which these enzymes have been altered. Instead, in *E. coli* two classes of mutations have been found by this selection technique (7, 17): (i) penicillinase producers and (ii) strains in which the structure of the cell envelope is changed so as to alter the permeability of the cell to penicillin. To investigate this problem, we have therefore devised a technique which, in part, may avoid the selection of these two classes of mutations. This technique and the classification of the mutants which have been isolated with it from *E. coli* are presented in the present paper. In particular, two interesting classes of temperature-sensitive mutants of *E. coli* were found in which the peptidoglycan of the cell wall synthesized at the nonpermissive temperature appears to be either hypo-cross-linked or hyper-cross-linked.

MATERIALS AND METHODS

Bacterial strains. The strain of *E. coli* used in this study was strain H 102, kindly given by J. Kirschbaum, Harvard University. This strain is a derivative of strain Hfr-H. To measure peptidoglycan synthesis conveniently in vivo, Dap$^-$ and Lys$^-$ mutations were introduced as follows. A mid-log-phase culture (5 ml, 3×10^8 cells per ml) was treated with 300 μg of

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N-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 15 min (1). The cells were collected by centrifugation and washed. The cell survival was 30%. These cells were plated on YAB medium (Difco yeast extract, 5 g/liter; and Difco antibiotic medium 3, 17.5 g/liter) and then replica-plated to minimal medium M 63 (20) with or without 20 μg of L-lysine per ml. The frequency of the Lys⁻ phenotype was 0.5×10^{-3}. One of these isolates was further mutagenized, as above, and plated on DYAB medium (YAB supplemented with 0.2 mM diaminopimelic acid [Dap]) from which it was replica-plated to minimal medium with 34 μg of lysine per ml and with or without 36 μg of Dap per ml. The frequency of the double mutant, Lys⁻, Dap⁻, was about 0.25×10^{-3}. One of these double mutants that grew rapidly either in nutrient broth or agar supplemented with Dap was used for further study and was designated strain H 2143. The generation time for this strain in broth was about 60 min compared to about 30 to 40 min for the parent culture, H 102, at 32 C, but at 42 C it grew with the same generation time as the parent, about 20 min. Another strain isolated in the course of this study is H 212. It is Lys⁻ and has a temperature-sensitive Dap⁻ mutation, requiring Dap at 42 but not at 32 C.

Assay of peptidoglycan synthesis and cross-linking in vivo. An overnight culture was grown at 32 C without shaking. A 0.1-ml sample was freshly inoculated into 2 ml of YAB medium to which [^{14}C]Dap was added. Both the inoculum and the fresh medium were prewarmed to the desired temperature before initiation of the experiment. After 2.5 h of incubation at either 32 or 42 C, 0.25 ml of 50% trichloroacetic acid was added and the sample was heated in a boiling-water bath for 5 min. Insoluble material was removed by centrifugation and washed with 2 ml of 0.1 M ammonium acetate; 60 μliters of 2 mg of trypsin per ml in 0.1 M ammonium acetate-0.01 M ethylenediaminetetraacetic acid (pH 7.9) was added; and the mixture was incubated at 37 C for 2 h. After heating in a boiling-water bath for 2 min, 40 μliters of 10 mg of lysozyme per ml in the same buffer was added, and the mixture was incubated at 37 C overnight. This material was then applied to Whatman no. 3MM filter paper and subjected to descending chromatography in isobutyric acid-1 N ammonium hydroxide (5:3) overnight. A radioautogram was prepared, and the areas corresponding to the peptidoglycan monomer (GlcNac-MurNac-tetrapeptide, R_f = 0.4) and the cross-linked dimer (R_f = 0.2, including very small amount of oligomer at an R_f = 0.1) were cut out and counted in the liquid scintillation spectrometer.

RESULTS

Isolation of temperature-sensitive ampicillin-resistant mutants. It was decided to isolate a class of mutants that would be resistant to penicillin at low temperature, 32 C, and which lysed in the absence of penicillin at 42 C. The rationale for isolation of this class of mutants was that penicillinase producers, which would be penicillin resistant at 32 C, would not lyse at 42 C and similarly that envelope mutations with altered permeability at 32 C might not lyse at 42 C. The two major classes of penicillin-resistant mutants encountered previously should be eliminated by this screening technique, except in the case of double mutants. It was hoped that one of the penicillin-sensitive enzymes might be altered in such a way that it would be resistant to ampicillin at 32 C and relatively stable, but unstable at 42 C, resulting in death of the cell. The isolation was carried out as follows.

Method 1. After treatment of 5 ml of culture with 200 to 300 μg of nitrosoguanidine per ml for 15 min, the cells were collected by centrifugation and washed. The cells were suspended in 5 ml of broth containing 5 μg of ampicillin per ml and grown overnight at 32 C. The resulting cells were plated on eosin methylene blue (EMB) agar (15) without added sugar. The plates were then incubated at 32 C until small colonies were formed, usually overnight. The plates were then shifted to 42 C for 6 to 9 h. Most of the colonies were white, but those that turned purple were isolated by picking and replating on DYAB agar. Most of these colonies were not total lysis colonies; presumably, if they were, they could not have been isolated by this method. In fact, none of the 60 mutants isolated by this method lysed in broth when the temperature was raised to 42 C.

Method 2. The mutagenized cells were plated on DYAB agar containing 10 μg of ampicillin per ml and grown at 32 C. These original plates were then replica-plated with velvet to EMB plates at 32 C which were then shifted to 42 C as described in method 1. All of the colonies from the EMB plate that had been incubated at 42 C (whether purple or not) were tested by transfer individually to DYAB agar at 32 and at 42 C. Three types of colonies were observed: (i) transferable at either 32 or 42 C (these were discarded); (ii) transferable at 32 C but not at 42 C (these are colonies which did not undergo total lysis on the EMB plate at 42 C but which were, nevertheless, temperature sensitive); (iii) not transferable at either 32 or 42 C (these were colonies which apparently underwent total lysis at 42 C on the EMB plate; they were recovered from the master plate at 32 C). A total of 481 mutants isolated by these two methods have been examined. An additional 38 mutants isolated in a similar way from broth containing 5 μg of cephalothin per ml were isolated, but since they did not differ significantly from the ampicillin-resistant strains they will not be considered further.

All of these mutants were tested after purifi-

cation by growth at 32 C on plates with and without 10 μg of ampicillin per ml, and at 42 C on DYAB plates, virtually all had the desired phenotype (ampicillin resistant at 32 C and no growth at 42 C). Next, each colony was grown in DYAB broth at 32 C overnight and, depending on the apparent density, 0.1 to 0.5 g/ml was transferred to 4 ml of fresh broth to give an estimated cell density of 10^7/ml. This culture was then incubated at 32 C for 90 min and then shifted to 42 C. After 3 to 4 h, the morphology of the culture was examined by phase-contrast microscopy. In addition, lysis was observed as clearing of the culture with some residual debris. Slow growth or no growth at 42 C was also noted as low density. A wide variety of morphological aberrations were observed, some of which are illustrated in Table 1 (and also in Table 4 and Fig. 3).

Assay of peptidoglycan synthesis and cross-linking in vivo. Initially, all of the cultures were examined by this technique at 32 and 42 C. The extent of peptidoglycan synthesis at both temperatures could be estimated from the total radioactivity incorporated, and the extent of cross-linking in the newly synthesized peptidoglycan could be determined at each temperature from the ratio of dimer to monomer. Moreover, the relative cross-linking at the two temperatures could be measured as the ratio of cross-linking (dimer/monomer ratio) at 42 C to the cross-linking at 32 C (normal range, 0.95 to 1.25). After about 100 cultures were examined in this way, it became apparent that the ratio of dimer to monomer at 32 C was about 0.9 and was itself relatively constant among all the cultures, the range being 0.8 to 1.0. To facilitate examination of additional cultures, it was therefore decided to examine only the ratio of dimer to monomer at 42 C (normal range 0.9 to 1.1), considering any value outside of the range of 0.8 to 1.2 as abnormal. Some representative examples are given in Table 1. All of these data are represented in Fig. 1. Duplicate determinations of this kind were highly reproducible. It can be seen from the representation in Fig. 1 that there is a small group of mutants in which the peptidoglycan synthesized at 42 C was hypo-cross-linked (defined as dimer/monomer ratio less than 0.8) and another group in which the peptidoglycan synthesized at 42 C was hyper-cross-linked (defined as a dimer/monomer ratio greater than 1.2).

Classification of the mutants. A number of criteria were employed to classify the mutants obtained: (i) extent of peptidoglycan synthesis at 32 and at 42 C, (ii) extent of cross-linking at 42 C, (iii) morphology at 42 C, (iv) extent of lysis or growth at 42 C. Three or four subdivisions were made in each class (Table 2), and each mutant was assigned a four-digit classification depending upon its properties. For example, mutant HAT 1 is classified 1214. (HAT: H, HfrH; A, ampicillin resistant; T, temperature sensitive). In this mutant, peptidoglycan synthesis at 32 C was normal, but was low at 42 C. At 42 C it synthesized hyper-cross-linked peptidoglycan. Its morphology at 42 C was spherical, and its growth at 42 C in liquid culture over a period of 3 to 4 h was normal (despite the fact that all these mutants were isolated as organisms that could not form colonies on plates at 42 C). Of the 481 mutants that were isolated, 194 were studied in detail and are classified in Table 2. There are several interesting groups of mutants.

Group 1: hypo-cross-linked HAT mutants. Fifteen of the mutants synthesized hypo-cross-linked peptidoglycan at 42 C (classification 112X or 113X). All of these had normal peptidoglycan synthesis at 32 C and markedly reduced peptidoglycan synthesis at 42 C, in addition to the low cross-linking of that peptidoglycan that was synthesized. None of them was spherical. Virtually all of them (11/15) lysed wholly or partially at 42 C (classifications 1121, 1122, 1131 and 1132) (Fig. 2B, compare to control [Fig. 2A] and Fig. 3A–C). A few showed characteristic bulges at the center of the rod. It is also noteworthy that none of these mutants had normal peptidoglycan synthesis at both temperatures (classification 31XX), and none of them had low peptidoglycan synthesis at 32 C as well as at 42 C (i.e., no mutants were in classification 21XX).

It should be noted that the effect of ampicillin on peptidoglycan synthesis and cross-linking in the parent culture at 32 C (Table 1) was similar to that observed in this group of mutants at 42 C. In addition to reducing cross-linking in vivo, ampicillin also reduced total in vivo peptidoglycan synthesis. Cross-linking, although reduced, was not totally inhibited in the peptidoglycan that was synthesized. This is in contrast to the effects of ampicillin on in vitro peptidoglycan synthesis where cross-linking is inhibited without any effect on total peptidoglycan synthesis (12). This difference occurs presumably because peptidoglycan synthesis and cross-linking are coupled by some kind of regulatory mechanism in vivo but not in vitro.

Group 2: hyper-cross-linked mutants. The organisms which synthesized hyper-cross-linked peptidoglycan at 42 C fall into two subgroups. Seven of the 12 mutants in this class had normal peptidoglycan synthesis at 32 C but

Table 1. Characterization of Mutants

HAT Mutant [Class]	On Plates				In Broth		In vivo assay of peptidoglycan synthesis				
		32C	42C		42C		[^{14}C]Dap Incorporation[a]		Dimer/Monomer		
	-	+Amp	-	Penicillinase Production	Cells[d]	Culture[c]	32C	42C	32C	42C	42/32C
Parental strain											
H2143	+	-	+	-	[b]	N	45	49	.93	.98	1.15
with 5μg/ml Ampicillin						L	11		.73		(.79)[e]
with 20μg/ml Ampicillin					[b]	L	2		.51		(.55)[e]
1. Hypo-Crosslinked											
HAT 436 [1122]	+	+	-	-		PL	35	8	.98	.77	.79
HAT 452 [1131]	+	+	-	-		L	29	1	.91	.79	.87
2. Hyper-Crosslinked											
HAT 276 [1214]	+	+	-	-		N	30	12	.94	1.41	1.50
HAT 20 [3224]	+	+	-	-		N	34	38	.94	1.26	1.34
3. Low peptidoglycan synthesis at both temperatures, Normal Crosslinked											
HAT 14 [2324]	+	+	-	-		N	17	13	.95	.97	1.02
HAT 85 [2322]	+	+	-	-		PL	9	9	.84	.91	1.08
4. Normal Crosslinked											
HAT 224 [1323]	+	+	-	-		LD	31	9	.90	1.03	1.14
HAT 141 [3334]	+	+	-	-		N	55	52	.89	1.10	1.24

[a] Total cpm x 10^{-3}

[b] At 32C with ampicillin

[c] N=Normal, L=Lysis, PL=Partial lysis, LD=Low density

[d] Cross hatching=ghosts of cell

[e] With ampicillin/control

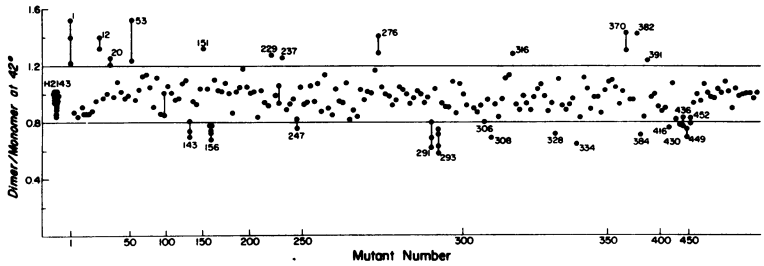

FIG. 1. *Dimer/monomer ratio in various strains at 42 C. Data points connected by vertical lines are duplicate determinations. See text for details.*

TABLE 2. *Classification of mutants*[a]

1st three digits of classification			Fourth digit of classification				Totals		
			1	2	3	4			
1	1	1	0	0	0	0	0		
		2	1	4	0	2	7	15	
		3	5	1	1	1	8		
1	2	1	0	0	0	7	7		
		2	0	0	0	0	0	7	80
		3	0	0	0	0	0		
1	3	1	0	1	1	2	4		
		2	3	10	3	19	35	58	
		3	6	7	0	6	19		
2	1						0		
2	2						0		
2	3	1	2	0	1	4	7		20
		2	0	2	1	5	8	20	
		3	0	2	0	3	5		
3	1								
3	2	1	0	0	0	0	0	0	
		2	0	1	0	4	5	5	
		3	0	0	0	0	0		94
3	3	1	0	0	2	3	5		
		2	2	2	0	60	64	89	
		3	3	0	1	16	20		

[a] **1st digit**—*peptidoglycan synthesis*: 1, low peptidoglycan synthesis at 42 C; <50% of 32 C and/or <25% of parent. 2, Low peptidoglycan synthesis at both 32 and 42 C; <25% of parent. 3, Normal. **2nd digit**—*cross-linking at 42 C*: 1, hypo-cross-linked; dimer/monomer <0.8. 2, Hyper-cross-linked; dimer/monomer >1.2. 3, Normal. **3rd digit**—*morphology at 42 C*: 1, spherical; 2, filamentous; 3, normal. **4th digit**—*growth in broth at 42 C*: 1, total lysis, i.e., clear lysis with many ghosts. 2, Partial lysis, i.e., low cell density with many ghosts. 3, Low cell density without ghosts. 4, Normal.

markedly depressed peptidoglycan synthesis at 42 C in addition to hyper-cross-linking at 42 C (classification 1214). All seven of these organisms were spherical at 42 C, and none of them lysed in broth at the high temperature (Fig. 2C, Fig. 3D, E). The other five hyper-cross-linked mutants (classification 32XX) had normal peptidoglycan synthesis at both temperatures.

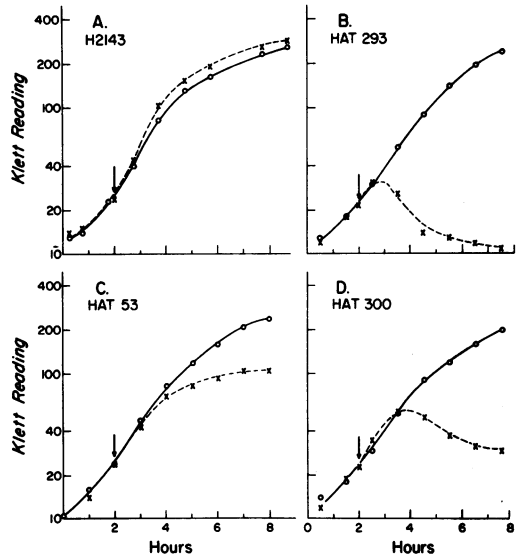

FIG. 2. *Effect of temperature shift on various strains. Growth was begun in duplicate cultures at 32 C. At the arrows the temperature was shifted to 42 C in one of the duplicates (dashed line).*

None of these was spherical and they formed either short or long filaments at 42 C (Fig. 3F) although they grew relatively normally in liquid culture over a 3- to 4-h period.

Group 3: mutants with defective peptidoglycan synthesis at both temperatures. The third group contains 20 organisms (classification 23XX). These were characterized by low peptidoglycan synthesis at both temperatures together with normal cross-linking. Many of these grew only slowly and gave low-density cultures at 42 C (8 out of 20); some were filamentous (Fig. 3G, H). This could represent a class of mutants in the membrane-bound enzymes of peptidoglycan synthesis, but they require much further study.

Group 4: mutants with normal cross-linking, mainly filament formers. The two largest groups of organisms (classification 13XX and 33XX) all had normal cross-linking and

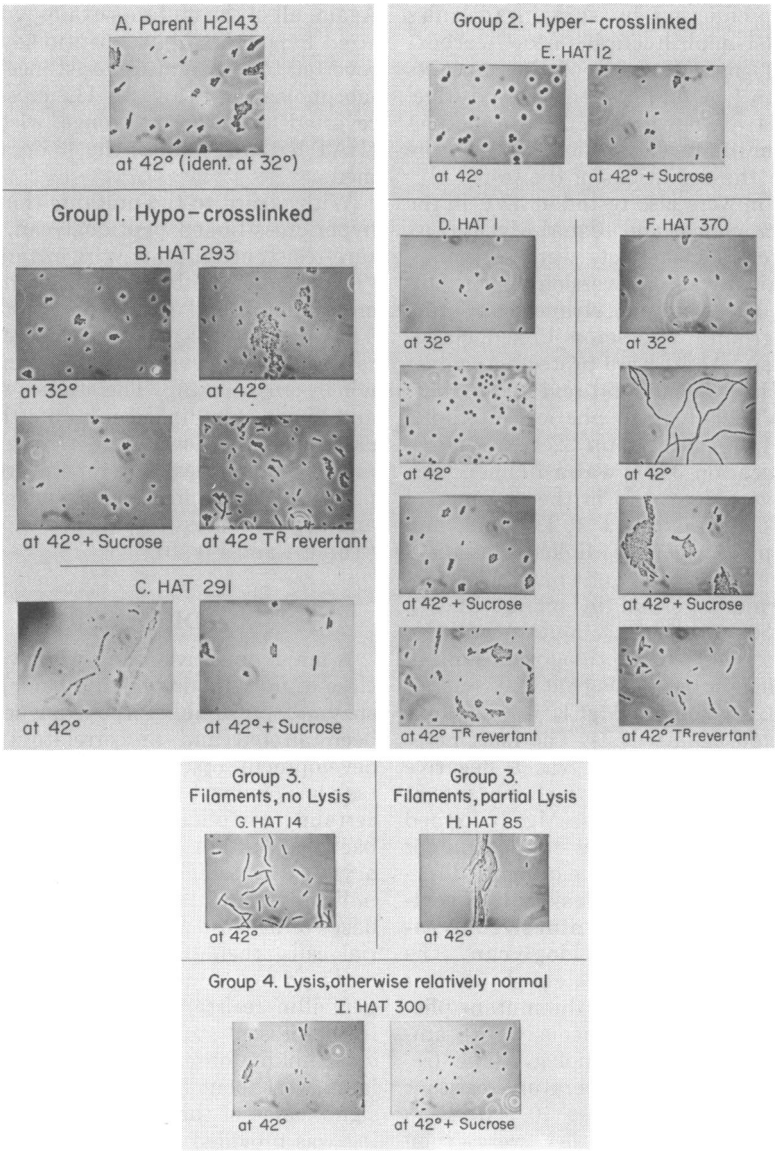

FIG. 3. *Phase-contrast microscopy of various cultures (×930). The conditions are indicated.*

were either normal in peptidoglycan synthesis at 42 C or low in peptidoglycan synthesis at 42 C. A very large percentage of these mutants formed short or long filaments at 42 C, and only a small percentage were true lysis mutants. There are three unusual organisms, however, (classification 3331) which had normal cross-linking, normal peptidoglycan synthesis, and normal morphology at 42 C, but nevertheless lysed (Fig. 2D, Fig. 3I). Because this group of organisms had normal cross-linking, they have not been investigated in any detail.

Two other points which should be mentioned are that among the mutants with low peptidoglycan synthesis at 42 C the organisms which synthesized hypo-cross-linked peptidoglycan at 42 C were mostly lysis mutants and none of them was spherical. The hyper-cross-linked mutants in this group were all spherical. Another interesting observation is that among the 52 HAT mutants which lysed in liquid culture at 42 C, wholly or partially (classifications XXX1 and XXX2), 70% had normal peptidoglycan synthesis at 32 C but decreased peptidoglycan synthesis at 42 C (classification 1XXX).

Many of these mutants were examined for

penicillinase production by spraying colonies with *N*-phenyl-1-naphthylamine-azo-*O*-carboxybenzene (19), including all of the mutants listed in Tables 1, 3, and 4. None was positive, in contrast to two known penicillinase producers, Amp A mutants (7), which gave purple colonies under the conditions of the test.

Many of them were also tested at 42 C in the presence of 20% sucrose and 10 mM $MgCl_2$ (Fig. 3). In all cases, some effects were seen, i.e., protection against lysis or reversion of morphology, or both. For example, strains HAT 156, 291, and 293 (group 1, hypo-cross-linked, classification 1121 and 1131) were protected against lysis; strains HAT 156 and 291 reverted to rods, but strain HAT 293 looked spherical or elliptical. Strain HAT 370 (group 2, hyper-cross-linked, classification 3224) was a filament former which reverted to rods in the presence of sucrose-$MgCl_2$. Strains HAT 1, HAT 12, and HAT 53 (group 2, hyper-cross-linked, classification 1214) were spherical organisms whose morphology was more normal in sucrose-$MgCl_2$. Strain HAT 300 was a lysis mutant with no apparent abnormality of peptidoglycan synthesis, cross-linking, or morphology at 42 C (classification 3331); in sucrose-$MgCl_2$ it was completely protected against lysis. The most noteworthy observation, however, was a negative one—no case was observed in which mutants at 42 C in the presence of sucrose-$MgCl_2$ formed spheroplasts similar to those formed under these conditions in the presence of penicillin.

Isolation of temperature-resistant revertants of the mutants which synthesized hypo- and hyper-cross-linked peptidoglycan. The purpose of this study was to establish whether the temperature sensitivity of the mutant phenotypes would revert simultaneously with ampicillin resistance and morphology. The frequency of spontaneous temperature-resistant revertants in both groups was 10^{-6} to 10^{-8}. Temperature-resistant colonies were then tested for sensitivity to ampicillin on DYAB plates containing 10 μg of ampicillin per ml. The frequency of ampicillin-sensitive revertants was mostly 0.5 to 15% of the temperature-resistant revertants. Detailed data for a number of these mutants are shown in Table 3. The double-reversion frequency was much greater than would be expected for two independent mutations, and it therefore appears likely that the temperature sensitivity and penicillin resistance were due to a single mutation.

A number of these revertants were also examined for reversion of morphology and for reversion of the abnormal cross-linking (Table 4). Again, all of the mutants examined that synthesized hypo-cross-linked peptidoglycan and had reverted to temperature resistance also reverted morphologically (Fig. 3). The cross-linking also reverted to the normal range, with 1 exception (HAT R 156-28) out of the 13 organisms examined.

With regard to the mutants that synthesized hyper-cross-linked peptidoglycan, 10 temperature-resistant mutants were examined in detail (Table 4). Of these, five had reverted to normal cross-linking and normal morphology (Fig. 3). The other five, although they were temperature resistant, had reverted neither in morphology nor in cross-linking. The studies of both hypo- and hyper-cross-linked mutants therefore indicated that there was a high correlation among mutant properties of temperature sensitivity, penicillin resistance, morphological change, and degree of cross-linking in the peptidoglycan (Table 4 and Fig. 3).

DISCUSSION

A major objective of this work was to isolate a class of mutants in which a penicillin-sensitive enzyme, presumably the transpeptidase, had been altered and to correlate this with the development of resistance to penicillin. This task has obviously been a difficult one, and it is certainly complicated by the finding that many bacteria contain multiple proteins which are acylated by penicillins (4, 5, 23, 24). If this fact indicates that there are multiple transpeptidases or at least multiple killing sites in bacterial cells, then the difficulty of isolating mutants in any one of them by selecting for penicillin resistance is immediately apparent. In the present study, the maximal reduction of cross-linking observed among the mutants isolated was about 30%. Some confidence in the significance of the 30% reduction of cross-linking was provided by the isolation of revertants in which cross-linking was restored together with morphology, temperature resistance, and penicillin sensitivity. It is noteworthy that no temperature-sensitive mutants could be found in which cross-linking at the nonpermissive temperature was very greatly reduced as occurs with ampicillin, and similarly no mutant was observed whose morphology at the nonpermissive temperature corresponded exactly to that produced by ampicillin.

An unusual and unexpected group of mutants which were isolated is a group in which the peptidoglycan synthesized at the nonpermissive temperature was somewhat hyper-cross-linked.

TABLE 3. *Reversion frequency*[a]

Mutant [Class]	Frequency of T^R	No. of T^R	No. of T^R which were Amp^s [b]	Frequency of co-reversion (%)
1. Hypo-cross-linked				
HAT 291 [1121]	$10^{-6} \sim 2 \times 10^{-8}$	48	2	4
HAT 436 [1122]	10^{-6}	200	0	<0.5
HAT 143 [1131]	10^{-7}	48	3	6
HAT 156 [1131]	10^{-8}	248	1	0.4
HAT 293 [1131]	$10^{-5} \sim 5 \times 10^{-7}$	1,128	4	0.4
HAT 449 [1131]	$10^{-5} \sim 5 \times 10^{-7}$	400	0	<0.3
HAT 452 [1131]	5×10^{-6}	200	1	0.5
2. Hyper-cross-linked				
HAT 1 [1214]	2×10^{-6}	48	7	15
HAT 12 [1214]	2×10^{-5}	200	2	1
HAT 53 [1214]	$10^{-5} \sim 5 \times 10^{-6}$	224	3	1.3
HAT 276 [1214]	2×10^{-6}	200	0	<0.5
HAT 370 [3224]	2×10^{-7}	150	3	2

[a] An overnight culture of each HAT mutant was plated on DYAB agar and incubated at 42 C for 1 or 2 days. Those spontaneous temperature-resistant (T^R) colonies which were obtained were purified on DYAB agar at 42 C and tested for their sensitivity to ampicillin on DYAB agar containing 10 μg of ampicillin per ml at 32 C.

[b] Amp^s includes some revertants that gave microcolonies on ampicillin plate. The majority (15/26), however, gave no growth at all.

These organisms were also penicillin resistant, and all of the unusual parameters of the mutants appeared to revert at the same time in some cases. The majority of these mutants was spherical. A number of other spherical mutations of *Escherichia coli* have been described (2, 9, 14, 16, 18). In two of these it has been established that the cell wall is not hyper-cross-linked. The other cases have not been examined from this point of view and, thus, we do not know whether the present mutation could be related to these.

It should be emphasized that the incorporation of [^{14}C]Dap in the various cultures was carried out for a relatively long period, and that more careful kinetic studies at much shorter time periods after shift up to 42 C would be necessary to establish a more definite relationship between the temperature sensitivity of the cultures and the alterations in cross-linking in these two groups of mutants. A number of possible explanations of these changes can be imagined in addition to alterations in amounts of transpeptidases. For example, a relatively slow rate of peptidoglycan synthesis while cross-linking remains at a normal rate can result in a hyper-cross-linked peptidoglycan, and similarly a relatively increased rate of peptidoglycan synthesis could result in hypo-cross-linking. The effects of endopeptidase and of D-alanine carboxypeptidase on cross-linking need to be considered, especially over a relatively long period of time during which some lysis is occurring. Increased activities of either could result in a hypo-cross-linked peptidoglycan and decreased activities could result in a hyper-cross-linked peptidoglycan.

An important question is: what is the basis for the ampicillin resistance of the various classes of mutants? Clearly, none were penicillinase producers. One could imagine, however, that some were "envelope" mutants with reduced permeability to penicillin at 32 C. Perhaps a fragile cell wall synthesized at 32 C was sufficient to permit normal growth of the organism, but that at a more rapid growth rate at higher temperatures the presence of such a cell wall could be lethal, either because the mutant structure was inadequate at rapid growth rates or because some essential component of it was denatured at the higher temperature.

Moreover, the relationship of growth rate to penicillin sensitivity is not defined precisely. It is known that non-growing cells are not killed by penicillin (10), and it therefore appears possible that slowly growing cells are less sensitive than rapidly growing cells. Thus, any mutation that results in a slowing of the growth rate may result in an increased resistance to penicillin. The group 3 mutants with low peptidoglycan synthesis both at 32 and at 42 C could be an example of this phenomenon since many of these mutants appear to be slow growers. In these cases, the rate of peptidoglycan synthesis at 32 C, although low, might be sufficient to maintain viability, but lysis at 42 C might then

Table 4 Characterization of revertants

HAT Mutant	On plates 32C -	On plates 32C +Amp	On plates 42C -	In Broth 42C Cells	In vivo assay of peptidoglycan synthesis [^{14}C]Dap Incorporation 32C	[^{14}C]Dap Incorporation 42C	Dimer/Monomer 32C	Dimer/Monomer 42C	Dimer/Monomer 42/32C
Parental strain									
H2143	+	-	+		103	110	.92	.95	1.08
1. Hypo-Crosslinked									
HAT 291 [1121]	+	+	-		64	3	.86	.69	.80
R291-1	+	±	+		52	65	.94	1.02	1.09
R291-4	+	-	+		49	36	.91	1.02	1.12
HAT 143 [1131]	+	±	-		45	8	.99	.74	.75
R143-2	+	-	+		90	70	.90	1.05	1.17
R143-16	+	±	+		53	19	.95	.96	1.01
HAT 156 [1131]	+	+	-		20	3	.95	.74	.78
R156-23	+	+	+		21	6	.99	.88	.89
R156-28	+	±	+		84	20	.84	.73	.87
HAT 293 [1131]	+	+	-		30	7	.94	.71	.76
R293-2	+	-	+		24	32	.74	.84	1.14
R293-3	+	±	+		49	65	.85	.90	1.06
R293-10	+	+	+		52	66	.87	.91	1.05
HAT 449 [1131]	+	+	-		28	1	.96	.69	.72
R449-1	+	+	+		28	42	1.02	1.05	1.03
2. Hyper-Crosslinked									
HAT 1 [1214]	+	+	-		70	35	.99	1.52	1.54
R1-1	+	±	+		126	92	.92	1.13	1.23
R1-2	+	+	+		97	85	1.02	.97	.95
HAT 12 [1214]	+	+	±		51	23	.98	1.32	1.35
R12-2	+	±	+		17	10	1.05	1.28	1.22
R12-3	+	-	+		70	35	.92	1.21	1.32
HAT 53 [1214]	+	±	-		58	25	.96	1.52	1.58
R53-1	+	-	+		54	42	.97	1.18	1.22
R53-2	+	-	+		79	64	.97	1.16	1.20
HAT 370 [3224]	+	+	-		37	66	1.10	1.31	1.19
R370-1	+	+	+		43	74	1.05	1.20	1.14
R370-2	+	-	+		67	100	1.07	1.25	1.17
R370-3	+	-	+		28	66	1.17	1.19	1.02

occur simply because peptidoglycan synthesis was unable to keep up to the rate of cell growth.

Thus, a number of possibilities for the phenotypic expression of penicillin resistance became apparent that were not obvious at the beginning of the study. Biochemical studies of the mutants are in progress with special attention to the group 1 (hypo-cross-linked) and group 2 (hyper-cross-linked) organisms in order to examine the question as to whether any of these mutants might contain lesions in transpeptidases or other essential enzymes of cell wall synthesis.

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