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Change in the Cell Envelope of *Escherichia coli* Carrying the Thermosensitive Drug Resistance Factor, Rts 1, at the Nonpermissive Temperature

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Escherichia coli, harboring the temperature-sensitive drug-resistant factor Rts 1, formed filaments on solid medium at the nonpermissive temperature (42 C). In addition, the rate of adsorption of T4D phage progressively decreased during growth at 42 C. Susceptibility to a variety of antibiotics increased suggesting that the permeability barrier to these antibiotics may be disrupted at the nonpermissive temperature. These observations were interpreted to suggest that the target of the temperature-sensitive Rts 1 gene product responsible for altering host growth may be the cell envelope.

The kanamycin (KM) resistance factor, Rts 1, is a thermosensitive plasmid which alters the growth of its host at the nonpermissive temperature (18). Previous experiments suggested that a gene product of Rts 1 deoxyribonucleic acid, present at both permissive and nonpermissive temperature, is altered at 42 C and results in abnormal cell growth. It has been shown that when Rts 1 cells were cultured at 42 C, total cell number increased linearly whereas viable cell numbers remained constant. This finding is consistent with asymetric cell growth at 42 C. Electron microscopy of these cells revealed membranous invaginations and the cells became susceptible to KM, as well as to actinomycin D. These observations suggested that the cell surface of Rts 1 cells (cells harboring Rts 1) was altered at the nonpermissive temperature (6). In this communication we present further evidence for this alteration. Filamentous cells were formed on a solid agar medium at 42 C. In addition, the kinetics of adsorption of phage T4 and susceptibility to various antibiotics were significantly altered at the nonpermissive temperature. Given the pleitropic nature of Rts 1 effect on host cells (6-8, 20), the structural and functional characteristics of the cell envelope make it a likely candidate as a target for the Rts 1 temperature-sensitive effector.

MATERIALS AND METHODS

Efficiency of phage T4D plaque formation. The R factor, Rts 1, the host strain *Escherichia coli* 20SO, and the conditions of culture have been described previously (6). Phage T4D was the generous gift of K. Takeishi. The efficiency of plating of T4D was measured as described (22, 21), with slight modifications. Bacterial cultures ([0.4 ml] 8 $\times$ 10^7 cells/ml) were adapted to 30 C by incubation for 2 min at this temperature. To 0.4 ml of culture was added 0.1 ml of a dilute phage suspension (1.4 $\times$ 10^3/ml) containing 100 μg of tryptophan per ml. After 10 min at 30 C, a 0.4-ml aliquot was withdrawn and transferred to a surface of a prepoured "bottom agar" plate containing 1.5% agar. Three milliliters of "top agar" containing 0.5% agar was melted, cooled to about 47 C, and poured onto the plates. After the soft agar layer had solidified, the plates were incubated at 37 C for 5 h. The extremely low multiplicity of infection eliminated the possibility of multiple infection of cells. The relative efficiency of plating was calculated by comparing the plaque counts on the various test lawns.

Kinetics of adsorption of phage T4D and assay for infectious centers. The methods described by Adams (1) were used to measure the kinetics of adsorption of phage T4D and titer the number of infectious-centers. Aliquots (0.5 ml) of bacterial cultures treated, as described in the appropriate figure legends, were mixed at 30 C with aliquots of suitably diluted phage suspensions. Tryptophan was added at a final concentration of 10 μg/ml. Samples of 0.1 ml were withdrawn at various times and diluted 100-fold into 10 ml of Trypticase soy broth-chloroform (9:1). After serial dilution in Trypticase soy broth, the dilution tubes were assayed for free phage by adding 0.1-ml aliquots to 2 ml of melted top agar at 47 C containing 0.2 ml of an exponential phase culture of *E. coli* 20SO R^- to provide a lawn of susceptible cells. This mixture was quickly poured over the surface of a prepoured bottom agar plate and incubated at 27 C overnight. The plaque counts were used to calculate the percentage of phage unadsorbed at various times relative to a control adsorption mixture (zero time sample) to which only phage were added. The rate of adsorption was expressed as: the velocity constant

k = (2.3/[cell concentration × time t] × log [free phage at t_0/free phage at time t]), in units of milliliters per minute. For the assay of infectious centers, 0.5 ml of the above adsorption mix was removed after 10 min of incubation and mixed with 0.1 volume of anti-T4D antiserum (k = 200, see reference 1) generously supplied by M. Dewey of this department. Unadsorbed phage were neutralized by incubation at 30 C for 6 min. After serial dilution, aliquots of 0.1 ml were mixed with *E. coli* 20SO R^- (*E. coli* 20SO not harboring drug resistance factors) to provide a lawn of susceptible cells. The plaque counts after incubation of plates overnight at 27 C were taken as the number of cells which adsorbed and produced phage.

RESULTS

Formation of filamentous cells. One indication that an alteration in the cell envelope may be linked to the Rts 1 effect on cell growth came from experiments in which Rts 1 cells were cultured on agar blocks containing glucose minimal medium. Blocks were seeded at low cell density to allow sufficient separation between individual neighboring cells. During incubation at 36 or 42 C, the blocks were examined periodically by phase-contrast microscopy to follow microcolony formation. During the 4 h of incubation almost every cell had given rise to a clone containing at least one filament (Fig. 1). All stages of microcolony development were found, up to the 8- or 16-cell stage. Some cells showed no growth at all (Fig. 1R). Other cells formed filaments without dividing (Fig. 1E, I, V). In contrast to Rts 1 cells at 42 C, control cells of Rts 1 at 36 C and R^- at 36 and 42 C showed

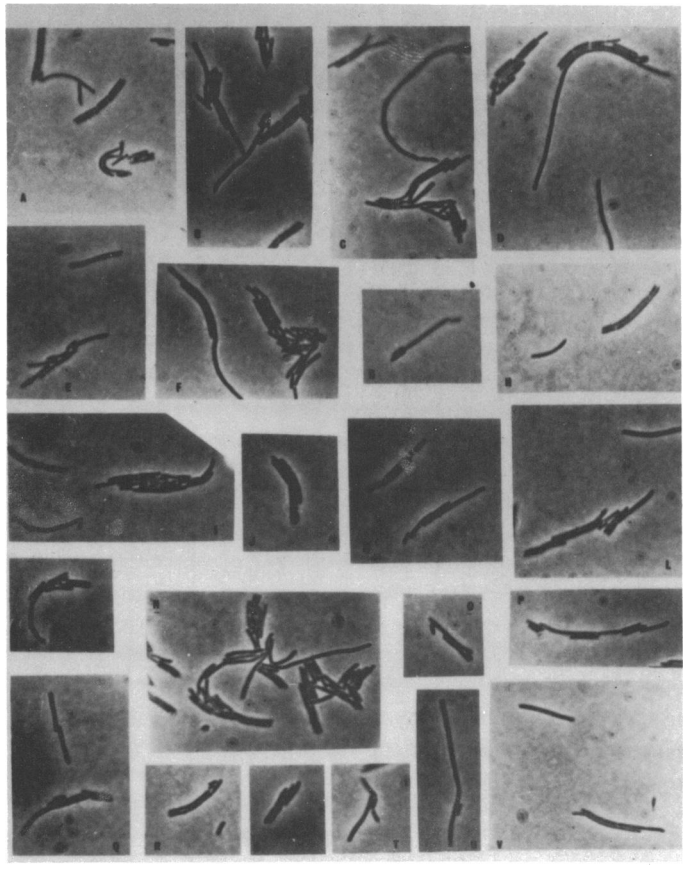

FIG. 1. *Growth of E. coli 20SO Rts1 at 42 C on solid medium. Exponential phase cultures of E. coli R^- and Rts 1 were diluted in glucose minimal medium and small volumes were placed on the surface of agar blocks. The blocks were prepared by pouring thin plates of glucose minimal medium agar and cutting squares (1 by 1 cm) with a sterile knife. The blocks were transferred to the surface of a sterile microscope slide inside a petri dish. A small vessel of water was placed in the dish to prevent drying of the agar during incubation. The block cultures were then incubated for up to 4 h at 36 or 42 C. After incubation, sterile cover slips were placed over the blocks, and they were examined and photographed under phase-contrast microscopy. Since filaments were only rarely seen in control cultures of R^- at 36 and 42 C and Rts 1 at 36 C, photographs were not taken. The figure shows various cell arrangements that were found after 4 h of incubation at 42 C.*

normal sized cells at the 8- to 16-cell stage, and filamentous cells were extremely rare (not shown). It should be noted that Rts 1 cells do not filament when cultured in broth at 42 C (6).

These observations suggest the possibility that Rts 1 may interfere with some structural or functional aspect of the host cell envelope. The formation of filaments on agar but not in broth might be due to a change in the phenotypic expression of the Rts 1 effect as a result of the particular osmotic conditions in the microenvironment of a cell growing on the surface of an agar block compared with broth.

Sensitivity of Rts 1 cells to various antibiotics. The R factor, Rts 1, confers resistance to KM. We have previously shown that Rts 1 cells show heightened sensitivity to KM when cultured in broth at the nonpermissive temperature (6). Table 1 shows that the ability of Rts 1 cells to form colonies on agar in the presence of KM is also dependent on temperature. In this experiment, cells which had been precultured at permissive temperature were used to inoculate MacConkey's lactose agar plates with and without KM. The number of colony-forming units was determined after incubation at different temperatures. There was no difference in plating efficiency on antibiotic free plates incubated at 25, 36, or 42 C, or on KM plates incubated at 25 or 36 C. This shows that each cell at the time of plating harbored the R factor. At 42 C, however, the efficiency of plating in the presence of KM was reduced over 90%. Thus, it appears that even though these cells harbored the R factor, many could not form a colony because of the detrimental effect of Rts 1 on host growth exaggerated by a heightened susceptibility to KM. We can rule out elimination of the R factor during growth of a colony on the plate since colonies formed at 42 C without KM were all resistant to KM when tested at the permissive temperature. The disk zone of inhibition method was used to determine if this heightened susceptibility extended to other antibiotics. Table 2 shows that Rts 1 cells at 42 C were sensitive to a variety of unrelated antibiotics, including chloramphenicol, erythromycin, and penicillin.

TABLE 1. *Plating susceptibility of E. coli Rts 1*[a]

Cells	Plating	Colonies/plate at:		
		25 C	36 C	42 C
Rts 1	+KM	160	163	14
Rts 1	-KM	167	168	169
R⁻	-KM	191	208	210

[a] A Trypticase soy broth culture of *E. coli* 20SO Rts 1 and R⁻ was diluted to approximately 1.5×10^3 cells/ml. Aliquots of 0.1 ml were spread over prepoured MacConkey's agar plates containing no KM or KM at 50 μg/ml. Plates were incubated at 25, 36, and 42 C for 2, 1, and 3 days, respectively, and the number of colonies which appeared on each plate was determined. Each number cited is an average of counts from 12 plates.

Response of Rts 1 cells to phage. As a probe for the change in the cell wall by Rts 1, we studied the response of R⁻ and Rts 1 cells to the phage, T4D. We first confirmed with modifications a finding of Yokota et al. (21) that restriction of T4 phage growth by Rts 1 breaks down at 42 C. In this experiment cultures of R⁻ and Rts 1 cells were incubated in glucose minimal medium for 4 h at 30 or 42 C. Phage were added at a very low multiplicity after normalizing cultures to the same optical density and equilibration to 30 C. After the adsorption period, cells and phage were plated to determine the efficiency of plating of T4D on these cells. As shown in Table 3, T4 phage plated with about equal efficiency on control cells cultured at 30 or 42 C, whereas the presence of Rts 1 at 30 C reduced the efficiency of plating about 600-fold. If Rts 1 cells were first precultured at 42 C, the efficiency of plating returned to about 25% of control values. Yokota et al. (21) interpreted this as a partial breakdown in an Rts 1 restriction system.

Yokota and Yamashita (personal communications) found that the total number of phage

TABLE 2. *Susceptibility of E. coli 20SO Rts 1 to various antibiotics*[a]

Antibiotic disk (μg)	Zone of inhibition (diameter in millimeters)			
	E. coli R⁻		*E. coli* Rts 1	
	30 C	42 C	30 C	42 C
Streptomycin, 2	0	0	0	15
Chloramphenicol, 5	10	10	10	29
Erythromycin, 2	0	0	0	19
Tetracycline, 5	12	12	12	17
Aureomycin, 5	12	12	12	18
Sulfadiazol, 0.25	0	0	0	0
Penicillin, 2 U	0	0	0	14

[a] Cultures of *E. coli* R⁻ and Rts 1 were diluted, and aliquots were spread over the surface of Trypticase soy agar plates to allow growth of a confluent lawn. Antibiotic-impregnated disks (Sensi-discs, BBL) were aseptically transferred to the surface of the plates. Incubation of duplicate plates was at 30 or 42 C for 1 to 2 days. The effect of each antibiotic was recorded as the diameter (millimeter) of the zone of inhibition of growth around the disk.

TABLE 3. *Efficiency of plating (EOP) of phage T4D on E. coli R⁻ and Rts 1 cells*[a]

Cells	Preculture temperature	Plaque count per ml	EOP
R⁻	30	4.75×10^9	1.00
R⁻	42	4.33×10^9	0.91
Rts 1	30	7.55×10^6	0.002
Rts 1	42	1.21×10^9	0.26

[a] Cultures of *E. coli* 20SO R⁻ and Rts 1 were inoculated at 10^7 cells/ml in glucose minimal medium. Portions of each culture were incubated at 30 or 42 C for 4 h. After normalization of each culture to the same optical density, the cultures were equilibrated for 2 min at 30 C. The plating of phage T4D was then performed by the procedures described in Materials and Methods. The plaque counts on each plate were used to calculate the concentration of phage in the original stock solution (column 3). The EOP is expressed as the plating efficiency relative to R⁻ at 30 C.

adsorbed in a long incubation was almost the same for Rts 1 versus R⁻ cells precultured at 42 C. However, possible differences in the rate of adsorption were not examined. We therefore measured the kinetics of adsorption of phage T4D to R⁻ and Rts 1 cells precultured for 4 h at either permissive or nonpermissive temperatures (Fig. 2). As shown in this figure, R⁻ cells precultured at 30 or 42 C, and Rts 1 cells cultured at 30 C, adsorbed phage at almost the same rate. However, there was a decrease in the rate of adsorption of about 50% of the control values after Rts 1 cells had been precultured at 42 C. Since the ratio of rates at 42 to 30 C was higher than the percentage of viable cells (30%), it was likely that some portion of the nonviable cells were adsorbing phage. Nevertheless, a change in adsorption properties was observed and this may reflect a change in the nature of the cell wall. The relationship among cell viability, adsorption, and phage production was examined by measuring the adsorption kinetics and numbers of infectious centers at various times after shifting a culture of Rts 1 cells to the nonpermissive temperature (Table 4). We

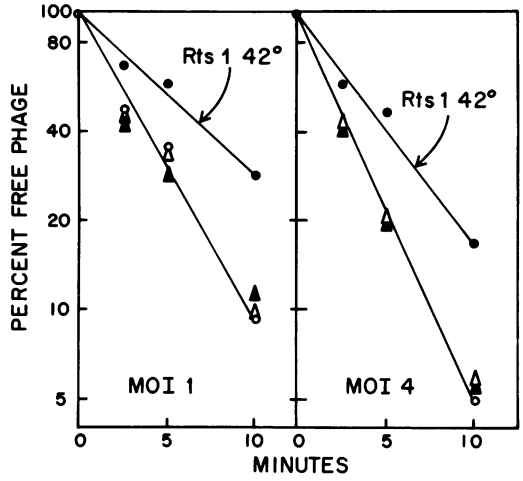

FIG. 2. *Kinetics of adsorption of phage T4D to cells with and without R factors. Cultures of E. coli 20SO R⁻ and Rts 1 were inoculated at 2×10^7 cells/ml in glucose minimal medium. Portions of each culture were incubated at 30 or 42 C for 4 h. After adjusting the cell density of each culture to 1.2×10^8 cells/ml, aliquots were equilibrated for 2 min at 30 C. Phage T4D was added at a multiplicity of infection (MOI) of 2 or 4. The kinetics of adsorption were assayed as described. The results are expressed as the percentage of free phage of input remaining at various times after addition to various cells precultured at the indicated temperature. Symbols: (Δ) R⁻, 30 C; (○) R⁻, 42 C; (▲) Rts 1, 30 C; (●) Rts 1, 42 C. The rate of adsorption, k, for Rts 1 cells at 42 C was 2.22×10^9 ml/min at MOI = 2 and 2.67×10^9 ml/min at MOI = 4.*

TABLE 4. *Changes in adsorption and production of phage T4D after a temperature shift to 42 C*[a]

Time (h)	Number of cells tested $\times 10^{-7}$	Viable count $\times 10^{-7}$	Percentage of cells viable	Rate of adsorption, k (ml/min $\times 10^9$)	Relative k	IC per ml	Relative IC
0	5.65	5.65	100	4.02	1.00	7.0×10^4	0.006
1	5.84	5.68	97	3.24	0.81	1.12×10^7	1.00
2	5.74	2.68	48	2.93	0.72	1.12×10^7	1.00
3½	5.79	1.59	28	2.50	0.62	5.60×10^6	0.50

[a] *E. coli* Rts 1 cells (5×10^7 cells/ml) grown at 32 C were shifted to 42 C in glucose minimal medium. At the times indicated, aliquots were withdrawn and equilibrated at 30 C for 2 min after adjusting the optical density to the level of the initial inoculum. The samples were then assayed for the kinetics of adsorption of phage T4D, phage production, and total and viable counts as described in Materials and Methods, using a multiplicity of infection of 2. The rate of adsorption was expressed in terms of the velocity constant, k. The relative adsorption rate was calculated relative to the zero time value, and the relative number of infectious centers (IC) was calculated relative to the 1 h −42 C value.

could then follow the changes in these parameters as a function of changes in the ratio of viable to nonviable cells. As shown in this table, 100% of the cells were viable at the time of the temperature shift. As expected, the rate of adsorption at this time was similar to R^- control cells (Fig. 2), but only about 0.1% of the cells could produce phage. After 1 h at 42 C, a decrease in the rate of adsorption was observed even though all cells retained viability and there was also a 160-fold increase in infectious centers over the zero time value. After 2 h of growth at 42 C, nonviable cells had accumulated to about 50% of the total cell numbers. There was a further decrease in the rate of phage adsorption. Since the adsorption kinetics began to decline before the appearance of nonviable cells, some change had occurred in the cell walls of the viable cells. A change may also have occurred in the nonviable cells because there was no direct relationship between the change in adsorption rate over time and the change in the percentage of cells viable. Since there was no decrease in cell surface area determined by microscopy measurement of cell size during exposure to 42 C (unpublished observation), the decline of K value can't be due to the cell surface area change. Furthermore, the number of infectious centers was constant during the period when the percentage of viable cells decreased 50% (compare 1 versus 2 h). The number of infectious centers at 3.5 h then dropped to 50% of the previous (2 h) value. It appears, therefore, that newly formed nonviable cells can adsorb phage and act as infectious centers, but if these nonviable cells remain at 42 C, they gradually lose the ability to produce phage. This interpretation is consistent with the previous observation that nonviable cells may retain their capacity to synthesize macromolecules at least for 60 min at the nonpermissive temperature (6). The possibility still remains that the viable cells after prolonged exposure to 42 C may lose their ability to produce phage.

DISCUSSION

Approximately 2.1 copies of the drug resistance factor Rts 1 (molecular weight, 126 million) can reside in *E. coli* 20SO and give a pleiotropic effect on host cells at 42 C. At this temperature the formation of covalently circular Rts 1 deoxyribonucleic acid was hindered and viable cell numbers do not increase while total cell numbers increase linearly. Electron micrographs of cells exposed to the nonpermissive temperature showed abnormal cell membranes (6–8). Recent evidence suggests that cyclic adenosine 5′-monophosphate may play some role in the thermosensitive step of covalently closed Rts 1 deoxyribonucleic acid formation (20).

As a continuation of these studies on the thermosensitive drug resistance factor Rts 1, we examined further the possibility that the cell wall may be altered by the Rts 1 temperature-sensitive gene product. A defect in some aspect of wall growth or septum formation could explain abnormal host cell growth caused by Rts 1 at the nonpermissive temperature. For example, the membranous invaginations in these cells as reported earlier (6) could be a consequence of the accumulation of normal membrane at a site of defective wall growth or septum formation. The observations described in this communication are consistent with the above concept. Thus, Rts 1 cells formed filaments during growth on agar suggesting that the septation process may be hindered by the action of the Rts 1 thermosensitive gene product. It is still possible that septa-like structures may exist within these filaments. This effect was, however, dependent upon a particular environment of the cell because the filamentous form could not be observed in liquid culture. A change in the osmotic environment has been reported to alter the phenotypic expression of a number of cell division mutants (2, 12, 13). Some cell division mutants accumulate membranous inclusions (11). Changes have been noted in the cell envelopes of a number of cell division mutants (4, 5).

At the nonpermissive temperature Rts 1 cells became sensitive to a number of antibiotics including KM. The increased susceptibility to KM is probably a reflection of an envelope change, since increased susceptibility to a broad spectrum of other unrelated antibiotics was also observed (Table 2). Previous experiments (6) showed that even cells cultured in broth at 42 C had decreased resistance to KM. As much as 30 μg of KM/ml were required to obtain 50% inhibition of cell growth indicating that resistance still remained. The increased susceptibility to antibiotics at 42 C cannot be attributed to decreases in metabolic activity because macromolecular synthesis, as well as the cell generation time of viable cells at 42 C, was comparable to that of 36 C. Similar changes in susceptibility to antibiotics was reported for mutants of *E. coli* and *Salmonella* with possible defects in the lipopolysaccharide portion of the cell wall (15, 16).

In addition, the kinetics of adsorption of phage T4 were different at the nonpermissive

temperature (Fig. 2, Table 4). An altered response of the host to T4D and other phage can also be correlated with changes in antibiotic susceptibility and/or lipopolysaccharide (9, 10). Terawaki et al. (17) reported that transfer of KM resistance was temperature sensitive. Conjugal transfer is a complex process requiring many R factor gene products (19), some of which must certainly interact with host envelope components. The most obvious interaction of this kind is the insertion of R pili into the cell envelope. The delta transfer factor changes the phage typing pattern of *S. typhimurium* host cells (3). A staphylococcal penicillinase plasmid determines the biosynthesis of phage attachment sites (14). Parallel studies on the rate of phage adsorption and the number of viable cells after the temperature shift to nonpermissive temperature indicated that the change in the cell envelope precedes the loss of viability. In addition, this experiment demonstrated that the breakdown in restriction upon the temperature shift is complete within 1 h at which time most of the cells remained viable. These observations suggest that the cell envelope may be one of the direct targets of the gene product.

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