A TEMPERATURE SENSITIVE PROTEIN IN OUTER MEMBRANE OF ESCHERICHIA COLI K-12 HARBOURING A TEMPERATURE SENSITIVE R PLASMID, Rts1

Yoshiyuki Kamio and Yoshiro Terawaki

Department of Bacteriology, Faculty of Medicine,
Shinshu University, Matsumoto, 390 Japan

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SUMMARY. A temperature sensitive protein (protein T) of molecular weight 80,000 was found to exist in the outer membrane of Escherichia coli K-12 strains harbouring a temperature sensitive R plasmid Rts1, by the two dimensional acrylamide gel electrophoresis. Protein T was not detected either in R⁻, pTW2 (a mutant of Rts1) cells or in XR27 cells in which Rts1 is integrated into the host chromosome. Protein T was detected only when the Rts1⁺ cells were grown at the lower temperature, and it disappeared from the outer membrane within 90 min by growing the cells at 43°C. The critical temperature at which protein T disappeared was approximately 39°C.

Genetic and molecular characteristics of a temperature sensitive R plasmid Rts1 have been investigated by Terawaki et al. (1-5) and DiJoseph et al.(6). The replication of Rts1 is temperature sensitive and is under the stringent control at 30°C in Escherichia coli as well as in Proteus mirabilis(2,3). In contrast, replication of pTW2, which is a mutant of the Rts1, is no longer temperature sensitive and found to be under relaxed control at 30°C (5). Besides these, Rts1 shows a detrimental effect on the host cell growth at 42°C, but pTW2 has lost this effect(1,4). Recently, Gudas et al. have found a protein (protein D) in the outer membrane of E. coli strain B, and they considered it as a substance related to the initiation of DNA replication(7). These findings led us to perform the chemical analysis of the cell envelope components, especially the proteins existing in the outer membrane of the cells with or without Rts1 (or pTW2) by using a two dimensional acryl-

Abbreviations: HEPES, N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid. TEMED, N,N,N',N'-tetramethylenediamine.

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amide gel electrophoresis. In this communication we report on the presence of a temperature sensitive protein detectable only in the outer membrane of the Rts1$^+$ cells which were grown at 30°C.

MATERIALS AND METHODS

Bacterial strains and R plasmids. Bacterial strains and R plasmids used were shown in Table 1.

Isolation of outer membrane. The procedure was essentially the same as described previously(8). The cells were usually grown in 1,000 ml of Penassay broth(Difco, PAB) at 30°C with shaking. When the cell density reached approximately to 4 x 10^8/ml (mid exponential phase), the cells were harvested by centrifugation at 4°C, washed twice with ice cold 10 mM HEPES buffer(pH7.4) and finally resuspended into 15 ml of the HEPES buffer containing 0.5 mg of deoxyribonuclease and ribonuclease. Then, the cells were disrupted in a French pressure cell at 1,200 kg/cm^2. Total membranes were collected by centrifugation in Hitachi 65P rotor for 1 h at 50,000 rpm and were suspended into 3 ml of HEPES buffer and applied to a discontinuous sucrose density gradient (8 ml of 2.02M, 28 ml of 1.44M, and 17 ml of 0.77M sucrose in this buffer). The gradients were centrifuged in Hitachi SW-25-2 rotor for 18 h at 19,000 rpm. Outer membrane, thus obtained, was purified by washing with 2% of Triton X-100 in 10 mM HEPES buffer(pH7.4) according to the Schnaitman's procedure(9).

Acrylamide gel electrophoresis. Sodium dodesyl sulfate(SDS) slab gel electrophoresis was performed as described previously(10). Two dimensional (first: isoelectric focusing, second: SDS) gel electrophoresis was carried out by essentially the same method as described by Ames and Nikaido(11). A typical protocol was given in the legend to Fig. 1. After electrophoresis the gels were stained with 0.0025% Coomassie brilliant blue in 25% isopropanol/ 10% acetic acid.

Chemicals. Acrylamide and N,N'-Methylenebisacrylamide were from Eastman Kodak Co.. TEMED, Ammonium persulfate, Riboflavin and Triton X-100 were from Wako Pure Chemical Industries LTD(Tokyo), Nonidet P-40(NP-40) was from Shell Chemical Co.. Urea was "Ultra Pure grade" from Schwarz/man or Wako Pure Chemical Industries LTD. SDS was obtained from Galard Schlesinger. Ampholines were obtained from LKB. The other chemicals used were of the best grade commercially available.

RESULTS

Electrophoretic pattern of outer membrane. Fig. 1 shows the two dimensional slab gel patterns of the outer membrane from XA 7012, XA7012 with Rts1 or pTW2 and XR27 in which Rts1 was integrated into the host chromosome. We found a unique spot of protein

Table 1. Bacterial strains and plasmids used.

Strain	Genetic character	Source & Reference
Bacterium		
E. coli K-12		
XA7012	F^- Δ*lac galE*	J. R. Beckwith
XA7012 XR27	Rts1 genome is integrated into the host chromosome	(4)
W677	F^- *lac leu thr thi*	
Plasmid		
Rts1	Km^r, Rep^{ts}, Tra^{ts}, stringent[a)]	
pTW2	Km^r, Rep^{tr}, Tra^{ts}, relaxed[a)]	obtained from XR27 (4)

a) Type of replication at 30°C.
Km: Kanamycin, Rep: Replication, Tra: Transfer, r: resistant
ts: temperature sensitive, tr: temperature resistant

(hereafter we designate it as protein T) with a molecular weight of 80,000 and having isoelectric point of pH 5.5. Protein T was found specifically in the Rts1$^+$ cells which were grown at 30°C (Fig. 1a). The spot, corresponding protein T, was detected neither in pTW2$^+$ cells, plasmid$^-$ cells nor XR27 cells (Fig. 1b-d). The four gels were identical with one another in their protein patterns except for the spot of protein T. Protein T was not detected in the inner membrane fraction. Similar results were obtained when W677 was used as the host cells of these plasmids (data not shown).

Disappearance of protein T from the outer membrane of Rts1$^+$ cells grown at the high temperature. It has been well known that growth of the Rts1$^+$ cells was remarkably inhibited when they were incubated at 42°C(1,12). Therefore, an attempt was made to determine the amount of protein T of the cells grown at 43°C. XA7012 (Rts1) were grown in 3,000 ml of PAB at 30°C, and at the mid ex-

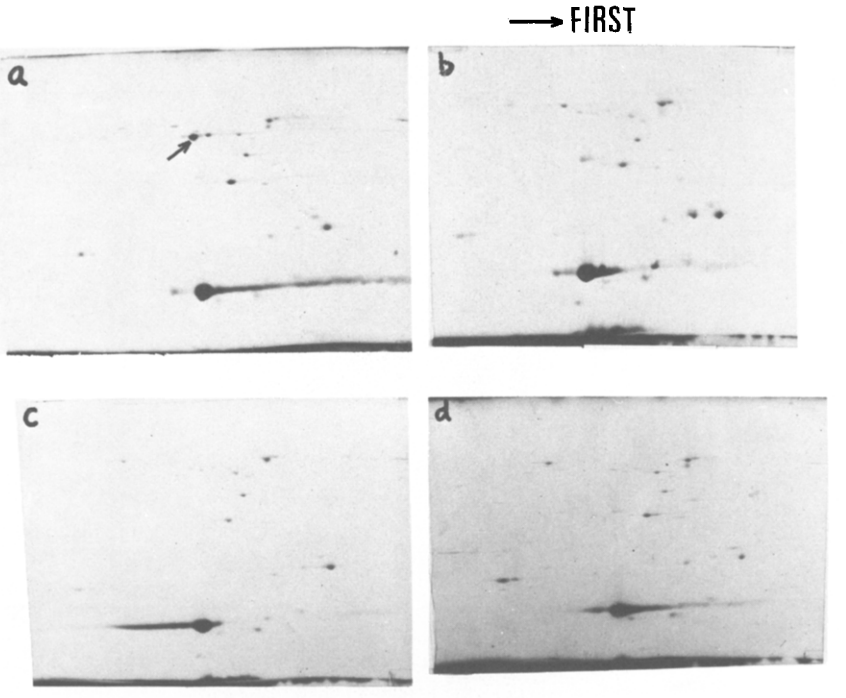

Fig. 1. Analysis of proteins in the outer membrane from XA7012 R^-, $Rts1^+$, $pTW2^+$, and XR27 cells by the two dimensional slab gel electrophoresis. Outer membrane was prepared from mid exponentially growing cells as described in Materials and Methods. Purified outer membrane was suspended into 1 mM Tris-HCl(pH6.8) (3 mg protein/100 µl). To 100 µl of the suspension were added 0.5 M Tris-HCl(pH6.8), 20 µl; 10% SDS(w/v), 40 µl; 0.1 M $MgCl_2$, 1 µl; water, 39 µl. After the incubation at 70°C for 30 min, it was centrifuged at 40,000 x G for 1 h. 100 µl of supernatant, containing the solubilized proteins, was diluted with two volume of sample dilution buffer which contained 9.5 M Urea, 2% Ampholines (comprising 0.4% pH3.5-10, 0.8% pH4-6, 0.8% pH6-8), 5% Mercaptoethanol(v/v) and 8% NP-40. 40 µg of the solubilized proteins, thus obtained, were applied to the first dimensional slab gels. The electrophoresis was performed at 300 V for 19 h, followed by 400 V for 1.5 h. In these gels the pH of the isoelectric focusing gel was 6.9 at the origin, 5.6 in the center, 4.2 at the front, respectively.
a. XA7012(Rts1). b. XA7012 XR27. c. XA7012. d. XA7012(pTW2)
——→ : protein T.

ponential phase the culture was shifted to 43°C. At time intervals, 1,000 ml of the culture was withdrawn, cells were harvested, and the proteins in the outer membrane were analyzed. As shown in Fig. 2, protein T disappeared almost completely from the outer

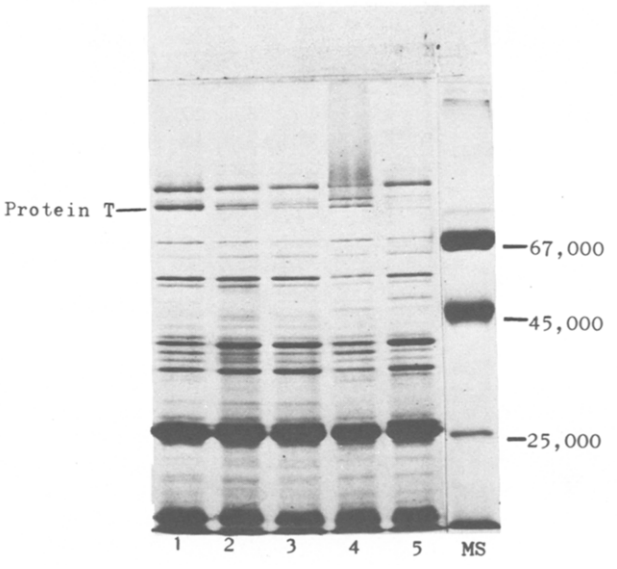

Fig. 2. Kinetics of protein T at the high temperature. XA7012 (Rts1) were grown in 3,000 ml of PAB at 30°C to mid exponential phase. Then the culture was shifted promptly to 43°C. Samples were withdrawn at intervals, and outer membrane was prepared as described in Materials and Methods. Purified outer membrane(3 mg) was solubilized by heating at 70°C for 30 min in 400 µl of "sampling buffer"(14), containing 5% mercaptoethanol and 2% SDS. 10 µl of each sample was applied to acrylamide slab gel (10%) containing 0.1% SDS. Molecular weight standards were: Bovine serum albumin (MW 67,000), hen egg albumin (MW 45,000), and chymotrypsinogen (MW 25,000).
1-3, Rts1$^+$: 0, 60, and 90 min after the shift, respectively.
4-5, R$^-$: 0 and 90 min after the shift.
MS, Molecular weight standards.

membrane within 90 min after the culture was shifted to 43°C. During the period, Rts1 cured cells did not exceed 5% of the total cell population, indicating that the disappearance of protein T should not be due to the overgrowth of Rts1$^-$ cells. Similar gel patterns were obtained when Rts1$^+$ cells were grown at 43°C for one (or two) hour even in the presence of 100 µg/ml of chloramphenicol. No quantitative differences were observed in the amount of protein T between the cells from mid exponential and late exponential phase culture if they were multiplied at 30°C.

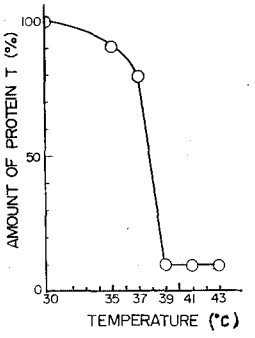

Fig. 3. Amount of protein T at various temperatures. XA7012 (Rts1) was grown in PAB at 30°C to mid exponential phase. Then the culture was divided, and each aliquot was shifted either to 35°C, 37°C, 39°C, 41°C, or 43°C. After 90 min of incubation, cells were harvested from each sample and outer membrane was prepared. After electrophoresis, the gel was stained and scanned by the densitometer (Shimadzu CS-90) by using 570 nm light length.

To determine the critical temperature at which protein T would disappear from the outer membrane, the following experiment was performed. The Rts1$^+$ cells were grown in PAB at 30°C and divided into several parts at mid exponential phase. Then, each aliquot was continued to grow at the various temperatures ranging from 30°C to 43°C for 90 min, and the outer membrane was prepared from each sample. The amount of protein T was calculated densitometrically. As shown in Fig. 3, the residual amount of protein T was 80% at 37°C and only 10% at 39°C, respectively. These facts clearly indicate that the critical temperature is approximately 39°C.

DISCUSSION

We have shown the presence of a novel protein in the outer membrane of E. coli K-12 cells carrying the Rts1 as a plasmid. This protein (protein T) disappeared from the outer membrane when the cells were grown at 43°C, with or without chloramphenicol. In addition, if the cells were shifted again to 30°C after the incubation at 43°C for 90 min, the amount of protein T recovered

within 90 min to the original level at 30°C. This recovery was inhibited completely when chloramphenicol was added to the culture (manuscript in preparation). These findings suggest a possibility that not only protein T is temperature sensitive, but also its synthesis is temperature sensitive.

Although the biological function(s) of protein T has not yet been investigated, it is certain that protein T does not play any role in the transfer of Rts1, restriction of phage T4, and antibiotic resistance, by the following reasons: i) Transfer of pTW2 is as temperature sensitive as Rts1. ii) Restriction of phage T4 is temperature sensitive in XR27 cells as well as in Rts1$^+$ cells (unpublished data). iii) Both pTW2 and Rts1 confer resistance to kanamycin/neomycin. Irrespective of these similarities, protein T was found neither in pTW2$^+$ cells nor XR27 cells.

Recently, protein D and protein G have been detected in the outer membrane of E. coli. The former is considered to be the substance for DNA replication(7) and the latter is for cell division(13). These evidences and ours mentioned above indicate that some of the proteins in the outer membrane are concerned with DNA replication and/or cell division, which are closely related phenomena to each other. The isolation and characterization of protein T are in progress.

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