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CUPRIC ION RESISTANCE AS A NEW GENETIC MARKER OF A TEMPERATURE SENSITIVE R PLASMID, Rts1 IN ESCHERICHIA COLI

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SUMMARY. A temperature sensitive kanamycin (Km) resistant R plasmid, Rts1, was found to confer cupric ion (Cu^{2+}) resistance on its hosts in Escherichia coli. At conjugal transfer, two kinds of segregants were obtained from Rts1, i.e. Cu^{2+} resistant, Km sensitive and Km resistant, Cu^{2+} sensitive plasmids. Protein T existed in E. coli cells harboring Rts1 or the $Cu^{r}Km^{s}$-plasmid. The inhibitory effect on the host cell growth at 43°C was observed with $Rts1^{+}$ or the $Km^{r}Cu^{s}$-plasmid^{+} cells. A relationship between these Rts1 derivatives and Rts1 in Proteus mirabilis which has been studied was discussed.

Rts1 is a kanamycin (Km) resistant R plasmid and its replication and conjugal transfer are temperature sensitive (1,2). In addition, Rts1 inhibits the host cell growth at 42°C (3,4). Our recent studies revealed the presence of protein T, a temperature sensitive protein,in the outer membrane of the $Rts1^{+}$ cells (5), and the production of extracellular DNase from the $Rts1^{+}$ cells (6) of Escherichia coli strains. We are intending to find the additional genetic markers other than Km on Rts1 genome which would allow to select easily the $Rts1^{+}$ cells. As a result we found a resistance to cupric ion (Cu^{2+}) on Rts1.

MATERIALS AND METHODS

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

Media for isolation and selection of $Rts1^{+}$ cells. M-9 glucose agar supplemented with amino acids (20μg/ml) and thiamine (2μg/ml) if required, was used as basal medium. When Km or Cu^{2+} resistance was transfered from XA7012 (Rts1) to W677, 0.1ml of the mixed culture was plated on the M-9 agar containing nalidixic acid (Nal: 50μg/ml) and either Km(50μg/ml) or $CuSO_4$ (1mM). In the mating between W677 R^{+} and JC1569, streptomycin (100μg/ml) was substituted for Nal.

Analysis of plasmid DNA in alkaline sucrose gradient. Cell lysate was prepared from the culture of JC1569 R^{+} cells labeled with 40μCi/ml of ^{3}H-thymidine by the procedure of Freifelder et al. (7). The lysate was layered onto a 5ml 5 to 20% linear alkaline sucrose gradient containing 0.5M NaCl, 0.02M EDTA and 0.3M NaOH, and centrifuged at 40,000rpm in a Hitachi RPS65T rotor for 18 min at 20°C. The fractions were collected from the bottom onto filter paper, washed with 5% trichloroacetic acid and counted in a Packard liquid scintillation counter.

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Isolation of outer membrane. Outer membrane was prepared by the methods as described previously (5).

Polyacrylamide gel electrophoresis. SDS-PAGE and two-dimensional gel electrophoresis were carried out as described previously (5) except the condition of the solubilization of the samples.

RESULTS

Transfer of Km and Cu^{2+} resistances. Stock culture of XA7012(Rts1) in Pennasay broth (Difco, PAB) was found to be composed of Km^rCu^r, Cu^r and Km^r cells, by the ratio of 75, 10 and 15%, respectively. The minimal inhibitory concentrations of $CuSO_4$ to XA7012 R^-, Km^r and Cu^r cells were 0.06mM, 0.06mM and 10mM, on the M-9 glucose plates. This finding led us to examine the transferability of Cu^{2+} resistance. XA7012(Rts1) resistant both to Km and Cu^{2+} was mated with W677 Nal^r at 30°C for 17h. The transconjugants were selected on the plates containing Nal and either Km or $CuSO_4$. W677 cells that received Cu^{2+} resistance formed yellowish brown colonies on the plate containing $CuSO_4$, whereas the Cu^{2+} sensitive cells could not grow on the plate. When Km was used for the selection, only 2% of transconjugants were resistant both to Km and Cu^{2+} In contrast, when the selection was made for the Cu^{2+} resistance, 97% of transconjugants received the both resistances. Thus we obtained three types of Rts1 plasmids at conjugal transfer, i.e. Rts1(KmCu), Rts1(Cu) and Rts1(Km). These three plasmids were transfered further from W677 to JC1569, and their integrity as plasmid was examined in this $recA^-$ host as follows.

Covalently closed circular (CCC) plasmid DNA. DNAs of JC1569 strains having either one of the plasmids were analyzed by alkaline sucrose density gradient centrifugation (Fig.1). Each cell lysate showed single peak which should be CCC-DNA of the respective plasmid. By cosedimentation studies with R28K ($44x10^6$ daltons) or NR1 ($63x10^6$ daltons), the molecular sizes of Rts1(KmCu), Rts1(Cu) and Rts1(Km) were estimated to be approximately $140x10^6$, $120x10^6$ and $100x10^6$ daltons., respectively.

Host cell growth at the non-permissive temperature. JC1569 cells with or without the plasmid were grown to mid-exponential phase at 30°C. Then the cells were diluted 100 fold into the fresh medium and continued to grow at ei-

Table 1. Bacterial strains and R plasmids

Strain	Genetic character	source
Bacterium		
E. coli K-12		
XA7012	F^- Δlac galE	J. R. Beckwith
W677	F^- lac leu thr thi Nal^r	laboratory strain
JC1569	F^- recA1 gal leu his Str^r	H. Uchida
W3104	F^- galT12	laboratory strain
R plasmid		
Rts1(KmCu)	Km^r Cu^{2+} r	this study
Rts1(Cu)	Km^s Cu^{2+} r	this study
Rts1(Km)	Km^r Cu^{2+} s	this study

Km: kanamycin, Cu^{2+}: cupric ion, Nal: nalidixic acid, Str: streptomycin
r: resistant, s: sensitive

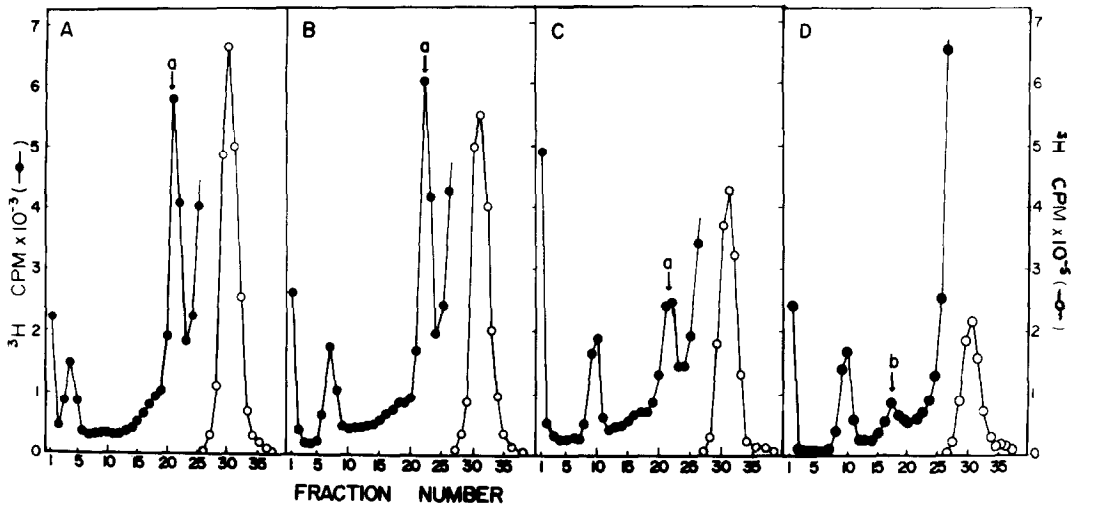

Fig.1 Alkaline sucrose gradient centrifugation of DNA from *E. coli* JC1569 (Rts1). Cells were grown in PAB containing 40uCi/ml of ^{3}H-thymidine and 250ug/ml of deoxyadenosine for at least two generations at 30°C. Cell lysates were prepared and analyzed in 5-20% alkaline sucrose gradients, as described in MATERIALS AND METHODS. In each centrifugation, lysate from *E. coli* W3104 (R28K) or W3104(NR1) was cosedimented with $Rts1^+$ cell lysate as a reference. (A) Lysates from JC1569(Rts1(KmCu)) and W3104(R28K). (B) Lysates from JC1569 (Rts1(Cu)) amd W3104(R28K). (C) Lysates from JC1569(Rts1(Km)) and W3104(R28K). (D) Lysates from JC1569(Rts1(Km)) and W3104(NR1). In the figure, a and b indicate CCC-DNA of R28K and NR1, respectively. Note the changes of scale at fractions 26-27. The large peak on the right (o-o) is sheared DNA of the host chromosome.

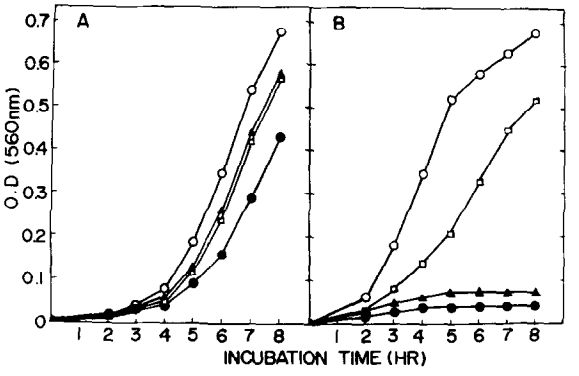

Fig.2. Growth of JC1569 R^-, Rtsl(Km)$^+$, Rtsl(Cu)$^+$ and Rtsl(KmCu)$^+$ cells. The cells were grown to mid-exponential phase with shaking in PAB at 30°C. Each culture was diluted 100 fold by fresh PAB and continued to grow at 30°C (A) and 43°C (B). Bacterial growth was followed by measuring optical density of the culture at 560nm with Coleman Junior II spectrophotometer. Symbols: ▲, JC1569 (Rtsl(Km)), □, JC1569(Rtsl(Cu)), ●, JC1569(Rtsl(KmCu)) and ○, JC1569 R^-.

ther 30°C or 43°C. As shown in Fig.2, although the growth of Rtsl(KmCu)$^+$ and Rtsl(Km)$^+$ cells were remarkably inhibited at 43°C, Rtsl(Cu) did not inhibit its host cell growth. Throughout these experiments the plasmid-cured cells were not detected at either 30°C or 43°C. Similar results were obtained in W677.

Protein T and other outer membrane proteins. Outer membrane proteins of XA7012, W677 and JC1569 cells with or without the plasmid were analyzed by SDS-PAGE (Fig.3). The W677 cells were analyzed further by two-dimensional gel electrophoresis (Fig.4). Protein T was found in all samples of the cells having Rtsl(KmCu) or Rtsl(Cu) (Fig.3). In regard to the "major proteins" (8) there was no difference among the four strains in W677 (Fig.4).

DISCUSSION

To our knowledge, this is the first report on plasmid mediated Cu^{2+} resistance in bacteria. Mechanisms of Cu^{2+} resistance has been reported previously in E. coli and yeast. In the former the resistance is ascribed to the disappearance of major protein b and c from the outer membrane, which inhibits penetration of the low molecular hydrophilic substances, including Cu^{2+} (9). In the latter, the Cu^{2+} resistance is due to production of a large amount of H_2S

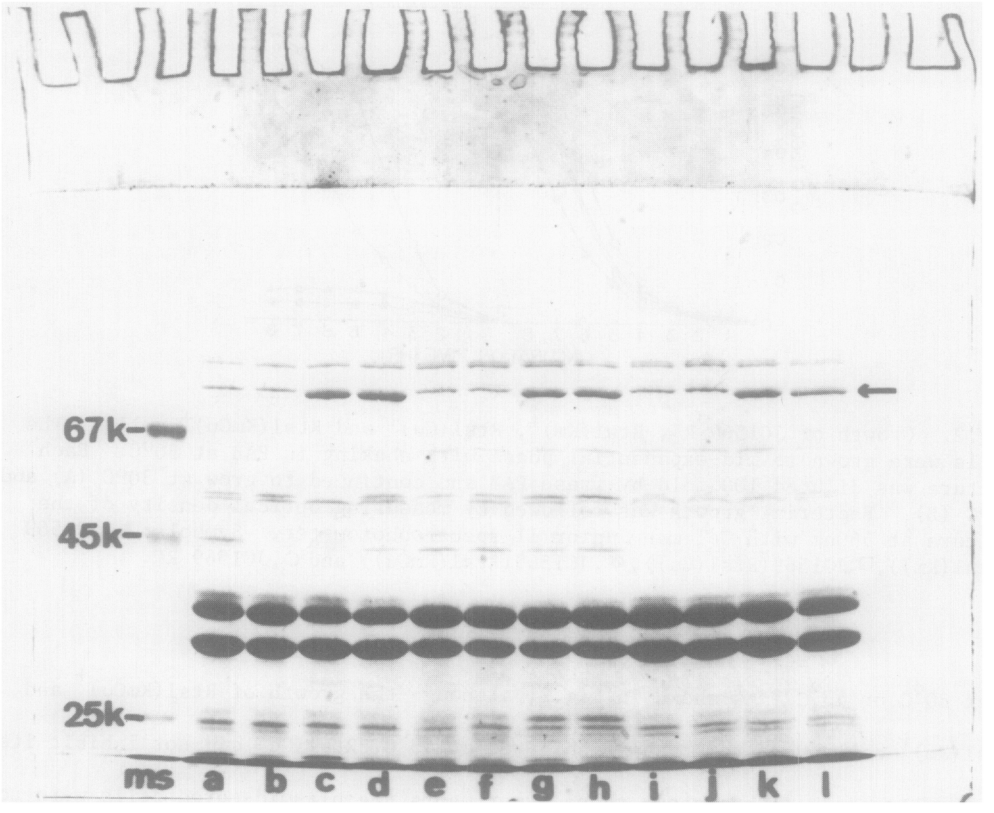

Fig.3. SDS polyacrylamide slab gel of outer membrane proteins from XA7012, W677 and JC1569 having either Rtsl(Km), Rtsl(Cu) or Rtsl(KmCu), or without R plasmid. Purified outer membrane was solubilized by heating at 100°C for 3.5 min in "sampling buffer" (5), and applied to acrylamide slab gel (9%), containing 0.1% SDS. Molecular weight standards were: Bovine serum albumin (MW 67,000), hen egg albumin (MW 45,000) and chymotrypsinogen (MW 25,000). (a) XA7012 R^-, (b) XA7012(Rtsl(Km)), (c) XA7012(Rtsl(Cu)), (d) XA7012(Rtsl (KmCu)), (e) W677 R-, (f) W677(Rtsl(Km)), (g) W677(Rtsl(Cu)), (h) W677(Rtsl (KmCu)), (i) JC1569 R^-, (j) JC1569(Rtsl(Km)), (k) JC1569(Rtsl(Cu)), (l) JC1569 (Rtsl(KmCu)), (ms) molecular weight standards. ⟶, Protein T.

that results in formation of harmless CuS. In addition, the resistance in yeast is lost when grown without Cu^{2+} (10). However the Cu^{2+} resistance by Rtsl(KmCu) and Rtsl(Cu) was stably maintained regardless of presence or absence of Cu^{2+}, and these cells resistant to Cu^{2+} had complete four major proteins in the outer membrane (Fig.4). Moreover, H_2S was not detected in the Rtsl$^+$ cells when tested in sulfide-indole-motility medium (data not shown). These findings suggest that the Cu^{2+} resistance of Rtsl might be due to a different mechanism from those described above.

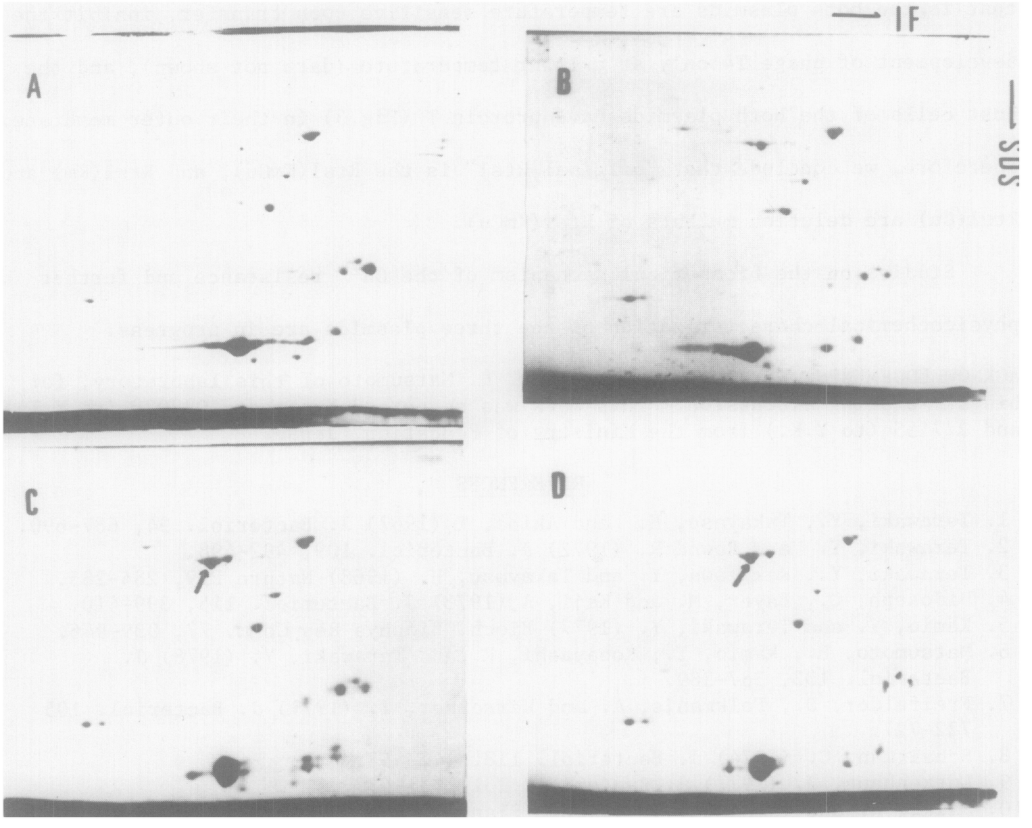

Fig.4. Two-dimensional slab gels of outer membrane proteins from W677 R^-, Rtsl(Km)$^+$, Rtsl(Cu)$^+$ and Rtsl(KmCu)$^+$ cells.
(A) W677 R^-, (B) W677(Rtsl(Km)), (C) W677(Rtsl(Cu)), (D) W677(Rtsl(KmCu)).
——→,Protein T.

As shown in Fig.1, the presence of three kinds of Rtsl molecules were confirmed in E. coli. This finding raises following two questions; i) Which molecule is the "usual Rtsl" that has been studied in this laboratory for long? ii) Was Rtsl(Cu) molecule really derived from "original Rtsl"? We have extensively studied the replication of Rtsl in Proteus mirabilis Pml7 (2,11,12). By a cosedimentation analysis of Rtsl in Pml7 and Rtsl(Km) in JC1569 in alkaline sucrose gradient centrifugation, the both plasmids were found to sediment at the same position, indicating that "usual Rtsl" corresponds to Rtsl(Km) plasmid. As to the second question, Rtsl(Cu) is considered to be a derivative of "original Rtsl", since Rtsl(Cu) shares several unusual properties in common with Rtsl(KmCu),

that is, the both plasmids are temperature sensitive upon transfer, inhibit the development of phage T4 only at a lower temperature (data not shown), and the host cells of the both plasmids have protein T (Fig.3) in their outer membrane. Therefore, we conclude that "original Rtsl" is the Rtsl(KmCu), and Rtsl(Km) and Rtsl(Cu) are deletion mutants of Rtsl(KmCu).

Studies on the biochemical mechanism of the Cu^{2+} resistance and further physicochemical characterization of the three plasmids are in progress.

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