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ALTERATION OF FLAGELLA BY A TEMPERATURE SENSITIVE R PLASMID Rts1 IN ESCHERICHIA COLI K-12

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SUMMARY. The presence of Rts1 and its mutants plasmids in Escherichia coli K-12 strains inhibited the motility of the host cells at 30°C. Aberrant flagella were observed with the plasmid$^+$ cells. Flagella protein (flagellin) obtained from the plasmid$^+$ cells did not co-electrophorese in polyacrylamide gel with that from plasmid$^-$ cells. Flagellin isolated from the cells harboring a mutant of Rts1, pTW10, gave two bands on polyacrylamide gel electrophoresis.

Genetic, molecular and biochemical characteristics of a temperature sensitive, Kanamycin (Km) resistant R plasmid Rts1 have been investigated by Terawaki et al.(1-5), DiJoseph et al.(6) and by us(7,8). Our recent study revealed that Rts1 conferred cupric ion(Cu^{2+}) resistance on its host in Escherichia coli, and that two kinds of segregants were obtained spontaneously from Rts1, i.e. Cu^{2+} resistant, Km sensitive (Rts1(Cu)) and Km resistant, Cu^{2+} sensitive (Rts1(Km)) plasmids(9). During a study of physiological characteristics of the three plasmids, we found that motility of the host cells in liquid medium and on nutrient gelatin agar plate was inhibited by the presence of Rts1 and Rts1(Km), but not by Rts1(Cu). In this communication we report on motility, flagellar shape and biochemical characteristics of flagella of E. coli K-12 cells harboring Rts1 or its mutant plasmid.

MATERIALS AND METHODS

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

Media. Nutrient broth (pH7.2) contained, per litter of distilled water: Bacto-Pepton (Difco), 10g; Beef extract (Difco), 3g; NaCl, 3g. Motility plates of nutrient gelatin agar (NGA) were prepared by adding 0.25% agar and 8% gelatin (Difco) to nutrient broth.

Isolation of flagella. The cells were grown in 300 ml of nutrient broth at 30°C with shaking. At mid exponential phase, cells were harvested by centrifugation at 6,000xG for 15 min at 4°C, and suspended in 20 ml of distilled water. Cell suspension was shaken in Homo blender (Sakuma Seisakusho, Tokyo) for one minute at about 12,000 rpm. Intact cells and debris were sedimented by centrifugation at 10,000xG for 15 min. Flagellar filaments were then pelleted by centrifugation at 37,000rpm for 45 min by Beckman 50 Ti rotor. Pelleted flagellar filaments were washed twice with distilled water. Purity of flagella protein(flagellin) was determined by SDS-PAGE. Flagellar filaments thus obtained were composed of 98-99% flagellin on total protein basis.

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Table 1. Bacterial strains and R plasmids

Strain	Genetic character	Source
Bacterium		
E. coli K-12		
XA7012	F⁻ lac gal E	J.R.Beckwith
JC1569	F⁻ recA1 gal leu his Str^r	H.Uchida
W3110	F⁻ hag207S(straight flagella)	J.Adler
W1485	F^+ supE42	
R plasmid		
Rtsl	Km^r Cu^{2+r} Rep^{ts} Tra^{ts} stringent[a)]	(9)
Rtsl(Cu)	Km^s Cu^{2+r} Tra^{ts}	(9)
Rtsl(Km)	Km^r Cu^{2+s} Rep^{ts} Tra^{ts} stringent	(9)
pTW10[b)]	Km^r Cu^{2+s} Rep^{ts} Tra^{ts} stringent	our laboratory

a) Type of replication at 30°C. b) A deletion mutant of Rtsl, obtained by N-methyl-N'-nitro-N-nitrosoguanidine treatment of $Rtsl^+$ strain (to be published). Str: Streptomycin. Km: Kanamycin. Rep: Replication. Tra: Transfer. r: resistant. s: sensitive. ts: temperature sensitive.

Preparation of flagellin. Flagellar filaments were disaggregated by 0.01N HCl and flagellin was obtained by the methods of Smith and Koffler(10). Amount of flagellin was determined with Lowry method(11) with bovine serum albumin as standard.

Polyacrylamide gel electrophoresis(PAGE). PAGE was performed by the method of Davis(12). SDS- and isoelectric focusing-PAGE were carried out as described previously(7).

Flagellar shape. Flagellar shape was examined electron microscopically. Cells were grown in nutrient broth. At mid exponential phase, glutaraldehyde was added to the culture to a final concentration of 2%. Then, fixed cells were centrifuged at 2,000xG for 10 min, washed twice with distilled water gently, and finally suspended into distilled water. Cell suspension was placed on Formvar-coated copper grid, and shadowed with chromium at an angle at 30°. Flagella were observed by electron microscope (Hitachi,HU-11A) using an accelerating of 75kV.

RESULTS

Motility in broth. Motility of JC1569 with or without plasmid was examined light-microscopically. Major type of movement was summarized in Table 2. As shown in this Table, R^- and $Rtsl(Cu)^+$ cells moved translationally. In contrast, the predominant type of movement of $Rtsl(Km)^+$ and $pTW10^+$ cells was a wriggle, and most of $Rtsl^+$ cells did not move, and a few rotated. If Rtsl or Rtsl(Km) was elininated from the host, the cells recovered normal motility. Similar results were obtained when W1485 was used as a host.

Spreading ability in NGA plate. JC1569 cells with or without the plasmid were sticked and grown on NGA plate for 18 hr at 30°C. As shown in Table 2 and Fig.1, $Rtsl^+$ cells could not spread on the plate at all. $Rtsl(Km)^+$ and $pTW10^+$

Table 2. Characteristics of E. coli with or without Rtsl and its mutant plasmids

Strain	Spreading ability[a)]	Movement in broth[b)]	Flagellar shape	Amount of flagellin(μg/mg dry weight of cells)
JC1569 R^-	1.0	Translation	Normal	15.7
JC1569(Rtsl)	0.0	No or rotation	Curly & coiled	9.2
JC1569(Rtsl(Cu))	1.0	Transration	Normal	19.9
JC1569(Rtsl(Km))	0.3	Wriggle	Straight(slightly helical)	9.5
JC1569(pTW10)	0.4	Wriggle	Curly	9.0
JC1569(Rtsl-cured)	1.1	Translation	Normal	14.3
XA7012 R^-	0.0	No or rotation	Curly	16.7
XA7012(Rtsl)	0.0	No	-	0.0
W1485 R^-	0.7	Translation	Normal	ND
W1485(Rtsl)	0.2	Wriggle & rotation	Curly	ND
W3110 R^-	0.0	No	Straight	13.1
W3110(Rtsl)	0.0	No	Straight(slightly helical)	12.5

a) Diameter of a swarm grown on NGA plate after 18h incubation at 30°C; the value of JC1569 R^- as 1.0.
b) We used nomencluture, described by Iino and Mitani(25).
-: No flagella, ND: Not determined.

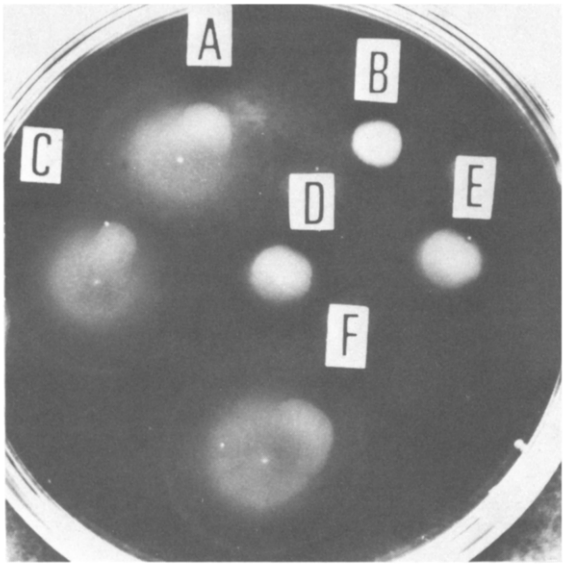

Fig.1. Spreading ability of E. coli JC1569 with or without Rtsl and its mutants on NGA plate.
A: plasmid$^-$. B: Rtsl$^+$. C: Rtsl(Cu)$^+$. D: Rtsl(Km)$^+$. E: pTW10$^+$. F: Rtsl-cured.

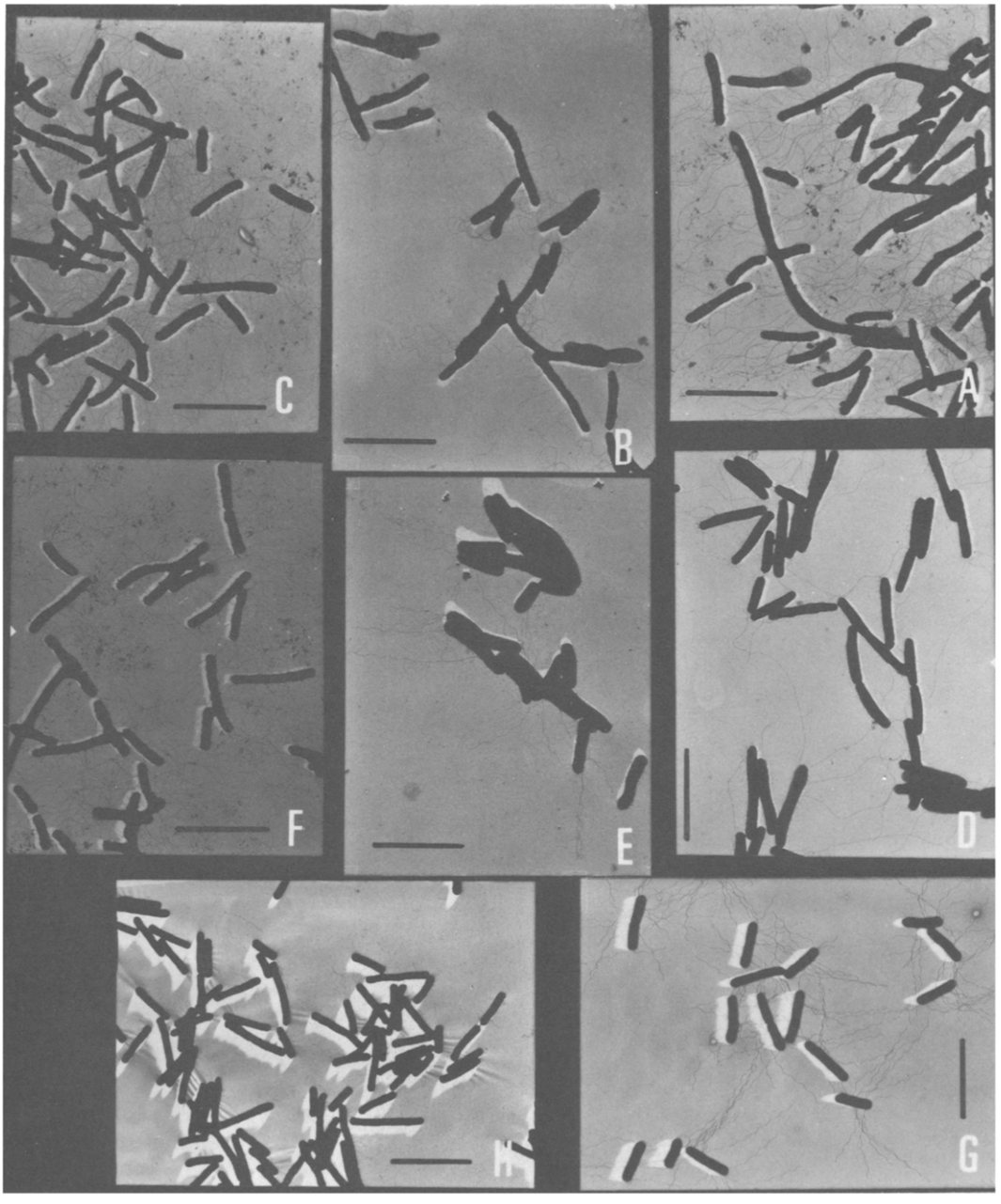

Fig.2. Flagellar shape of the representative strains with or without Rtsl and its mutants.
A: JC1569 R⁻. B: JC1569(Rtsl). C: JC1569(Rtsl(Cu)). D: JC1569(Rtsl(Km)). E: JC1569(pTW10). F:JC1569(Rtsl-cured). G: XA7012 R⁻. H: XA7012(Rtsl). Magnification bars represent 5 μm.

cells spread more slowly than plasmid$^-$ cells. On the other hand, Rtsl(Cu)$^+$ or Rtsl-cured cells had a same spreading ability as plasmid$^-$ cells.

Flagellar shape. Flagellar shape of the cells with or without the plasmid was examined electron microscopically. As shown in Fig.2, R$^-$ and Rtsl(Cu)$^+$ cells of JC1569 had normal flagella with wavelength of 2.1-2.2 μm and with amplitude of 0.5 μm. On the contrary, short, curly and coiled flagella were observed with Rtsl$^+$ cells. In Rtsl(Km)$^+$ and pTW10$^+$ cells, straight (slightly helical) and curly flagella were observed, respectively. Furthermore, an interesting finding was obtained when XA7012 was used as a host. The flagella of XA7012 was originally curly shaped, that is, its wavelength was 0.9-1.0 μm. If Rtsl was introduced into the cells any flagellum was not detected (Fig.2 H).

Amount of flagellin. As shown in Table 2, the amount of flagellin per unit dry weight of the cells varied considerably depending on the presence or absence of plasmid. The amount of flagellin from JC1569 cells with either Rtsl$^+$, Rtsl(Km)$^+$ or pTW10$^+$ was 60-70% of that from plasmid$^-$ cells. No flagellin was detected from XA7012(Rtsl) cells.

Biochemical characteristics of flagellin from the cells with or without plasmid. Flagellin obtained by disaggregation of flagella from the cells was analyzed by PAGE, SDS- and isoelectric focusing-PAGE. In regard to the molecular weight of flagellin, there was no difference among the cells with or without plasmid(Fig. 3 A). On PAGE, however, flagellin which was isolated from the cells with either Rtsl, Rtsl(Km) or pTW10 showed different migration on PAGE in comparison with that from plasmid$^-$ cells (Fig.3 B). Flagellin from Rtsl(Cu)$^+$ or Rtsl-cured cells migrated coincidently with that from plasmid$^-$ cells. It is interesting to note that flagella isolated from JC1569 with pTW10 gave two bands on PAGE, one corresponding to that from Rtsl(Km), and the other migrated slightly faster than that from plasmid$^-$ cells.

The isoelectric point of flagellin from Rtsl$^+$ cells was 4.74 and the values from all the other type of JC1569 were 4.82(Fig.3 C).

DISCUSSION

We have shown in this study that the motility of *E. coli* cells in broth and their spreading ability on NGA plate were remarkably inhibited by the presence of Rtsl, Rtsl(Km) and pTW10 at 30°C, and that amount of flagellin and number of flagella decreased in these cells. Furthermore, the aberrant flagella were observed in Rtsl$^+$, Rtsl(Km)$^+$ and pTW10$^+$ cells, and their flagellin molecules were different from that of plasmid$^-$ cells (Fig.3 B).

Hesslewood et al. reported that in *Proteus milabilis*, an R-TEM plasmid reduced swarming ability of its host cells(13). Skarr et al. reported that F$^+$ cells were less motile than the cells without the plasmid(14). However, they

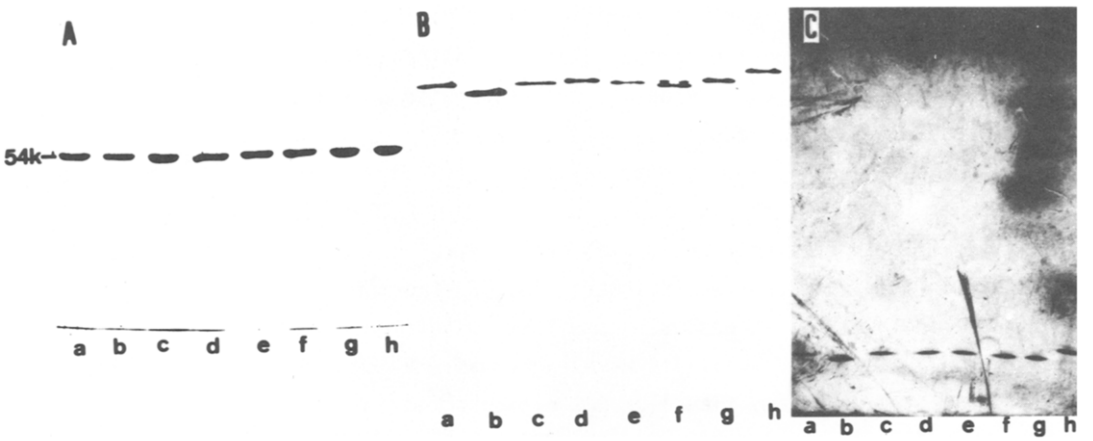

Fig.3. Acrylamide slab gel electrophoresis of flagellin obtained from JC1569 with or without Rtsl and its mutants.
A: SDS-PAGE(10% of acrylamide gel was used.). B: PAGE. The electrophoresis was performed at 20 mA until marker dye reached to the bottom. Flagellin was migrated from top to bottom towords the anode. C: Isoelectric focusing-PAGE. The pH of the gel was 7.5 at the origin (top of the gel), 5.8 in the center, 4.3 at the front, respectively. The gradient was linear. The dark area of this gel is due to the ampholines.
a: JC1569 R^-. b: JC1569(Rtsl). c: JC1569(Rtsl(Cu)). d: JC1569(Rtsl(Km)). e: JC1569(Rtsl-cured). f: JC1569(pTW10). g: XA7012 R^-. h: W3110(*hag*(S)) R^-. 54K→: Molecular weight of flagellin (54,000) of *E. coli*.

did not referred to flagella of the strains. Relationships between flagellar formation and synthesis of certain substances are reported. i.e. i) Cyclic AMP is absolutely requied for flagellar synthesis. When cyclic AMP(10^{-3}M) is added to the culture of cyclic AMP-deficient mutants of *E. coli* or of *Salmonella typhimurium*, flagellar formation of these strains and their motility are recovered(15). ii) Cellular content of ubiquinone or hemA is strongly related to flagellar formation and motility. Mutants of *E. coli* lacking ubiquinone or heme is immotile, and this is due to the loss of flagella(16). However, the decreased motility of $Rtsl^+$ cells was not rescued by adding cyclic AMP(10^{-3}M) and δ-amino levulinic acid (intermediate of hemA)(10^{-3}M) to the culture. In addition, concentration of ubiquinone in $Rtsl^+$ cells was at the same level with that of plasmid$^-$ cells. Recently, Komeda et al. have reported that *galU* mutants of *E. col* were immotile because of the loss of flagella(17). As *galU* mutants are known to be resistant to P1 phage(18,19), sensitivity of XA7012(Rtsl) to the phage was tested. XA7012 cells, irrespective of the presence or absence of Rtsl, were equally sensitive to P1 phage. We conclude from these findings that the loss of

motility of Rtsl$^+$, Rtsl(Km)$^+$ and pTW10$^+$ cells is not due to decrease in the amount of these substances, but due to the formation of aberrant flagella.

It is known that the mutants of E. coli and Salmonella defective in motility classified into the following types(20,21): i) Paralyzed mutants, which have normal flagellar shape but "paralyzed" because the flagellar filaments do not rotate. ii) Flagellin mutants which have altered flagellar configuration. This results from a mutation in a structural gene (hag) for flagellin. iii) Non flagellated mutants, which have no apparent flagellar structure. The Rtsl$^+$, Rtsl(Km)$^+$ and pTW10$^+$ cells resemble to the second type phenotypically. Martnez et al. isolated straight flagella mutants (not motile) from Bacillus subtilis, and they revealed that an alanine redidue in a polypeptide of normal flagellin in replaced with valine(22). Similar observation is shown in a curly flagella mutant of Salmonella, of which flagellin has a single peptide alteration(23). Our finding that Rtsl$^+$, Rtsl(Km)$^+$ or pTW10$^+$ cells had aberrant flagella, being composed of the altered flagellin, is consistent with their results.

We would mention that flagella isolated from pTW10$^+$ cells gave two bands of flagellins on PAGE. Although the mechanism of flagellar formation in pTW10$^+$ cells has not yet been investigated, the possibilities, suggested by Silverman and Simon(24), are applicable to our case. i.e. i) One type of flagellins might be segregated into one filament, and the other type might be segregated into a separate filament. ii) Two different flagellin subunits might be co-polymerized in blocks. iii) They might be intermixed and co-polymerized. The mechanism of assembly of flagellins in flagellar filaments which are synthesized by merodiploid strains (having two hag loci) of E. coli is examined by Silverman and Simon(24). They show that two kinds of flagellin molecules are intermixed and co-polymerized before being assembled into cell membrane system.

Further investigation is required for our study to distinguish among the three possibilities.

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