Colonization of R Plasmid-bearing *Escherichia coli* Strains in the Mouse Alimentary Tract

Kouji SHIMODA, Takatoshi KUHARA*, and Kazuyoshi MAEJIMA

Laboratory Animal Center, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160 and *Teikyo University School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173, Japan

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In order to examine the ability of R plasmid-bearing *Escherichia coli* strains to colonize in the mouse alimentary tract, an R plasmid-positive (R(+)) *E. coli* strain and its R plasmid-negative (R(-)) counterpart were together inoculated into the streptomycin-treated mouse alimentary tract, and the numbers of fecal *E. coli* strains were enumerated. The numbers of R(+) strains were always at the level similar to or lower than those of their counterparts and rapidly decreased in the fecal population. However, when R plasmids, which were originated from a cryptic plasmid of the host *E. coli* strain, were utilized, an R(+) strain dominated over its R(-) counterpart during the experimental period. These experimental results indicated that the relationship between the host strain and R plasmids affected the ability of the host strain to colonize in the alimentary tract. —KEY WORDS : alimentary tract, colonization, *Escherichia coli*, mouse, plasmids

Most of laboratory animals in Japan are free of drug-resistant organisms because of a principle of avoiding the use of antibiotic agents on animals for research [7, 13]. However, routine administration of antibiotics for prophylactic purpose has resulted in an increase in drug-resistant organisms and R plasmids in colonies of laboratory mice and rats [13]. Antibiotic agents are expected to apply more frequently to laboratory animals because proper treatments such as administration of appropriate antibiotics for prevention of suppuration after surgical experiments should be required from the viewpoint of animal welfare. Laboratory animals seem now to be in a situation somewhat comparable to an early stage of the appearance of drug resistant organisms in man and domestic animals.

On the other hand, many drug-sensitive *Escherichia coli* possess several cryptic plasmids, at least one of which was shown to be converted into conjugative R plasmids by translocation of drug-resistance transposons [11, 12], suggesting that some of these cryptic

plasmids play a role in the emergence and the prevalence of R plasmids. Although the significance of cryptic plasmids in drug-sensitive *E. coli* strains is still unknown, it seems that the relationship between host organisms and their cryptic plasmids is not of parasitism but of symbiosis. In general, *E. coli* strains carrying R plasmids become predominant in the fecal flora under the pressure of antibiotic agents, but after removal of the antibiotic pressure R plasmid-bearing (R(+)) strains disappear rapidly from the animal gut [1, 14]. The existence of cryptic plasmids in many drug-sensitive *E. coli* strains that are predominant in the fecal population seems to be inconsistent with the poor ability of R(+) strains for colonization in the intestinal flora. To determine whether possession of R plasmids is a negative factor for the ability to colonize in the animal intestine, we inoculated an R(+) strain and its R plasmid-free (R(-)) counterpart together into the streptomycin-treated mouse alimentary tract and examined their viable numbers in fecal samples.

Materials and Methods

Bacterial strains: Bacterial strains used in the present study were *E. coli* C SmRif, *E. coli* C 25 SmRif and *E. coli* cE 916 SmRif, *E. coli* C SmRif is a streptomycin-resistant mutant of *E. coli* C (a rifampicin-resistant derivative of K-12). *E. coli* C 25 SmRif is a rifampicin-resistant mutant of *E. coli* C 25 (a streptomycin resistant strain isolated from a mouse) [6]. To obtain cE 916 SmRif, we isolated a streptomycin-and rifampicin-resistant mutant from a drug-sensitive mouse strain E 916 [11], which possessed 5 cryptic plasmids, and pKO916Km was transferred into the mutant (E 916 SmRif) by transconjugation. After the cryptic plasmid, pKO916, originally existing in E 916 was supplanted by pKO916Km during several generations of cultivation under kanamycin selection, resulting kanamycin-resistant E 916 SmRif which carried pKO916Km instead of pKO916 was treated with ethidium bromide (50 µg/ml). A cured strain showing kanamycin-sensitivity was designated as cE 916 SmRif, which possessed 4 cryptic plasmids but not both of pKO 916 and pKO916Km (26 Md and 29.5 Md, respectively, data not shown).

R plasmids: R plasmids used in this study were listed in Table 1. These R plasmids did not possessed colicine factors. RP 4, R 144 and R 6 K are standard plasmids for the incompatibility test. pKO916km and pKO916Ap, which were originated from a cryptic plasmid pKO 916 in E 916, were obtained by translocation of drug-resistance transposons [11, 16]. Each R plasmid was transferred into R (−) strains by mixed cultivation with *E. coli* ML 1410 (a nalidixic acid-resistant F[−] derivative of K-12) carrying R plasmids as a donor.

Mice: ICR mice (CLEA Japan, Inc.) received 5 mg/ml of streptomycin sulfate (Sigma Chemical Co., USA) in their drinking water *ad libitum* for the entire experimental period beginning at 2 days before oral inoculation of organisms. Then, R (+) and R (−) strains, approximately 10⁷ organisms for each, were together given to the mice intragastrically. Fecal samples were collected periodically and after serial dilutions cultured on DHL agar plates (Eiken Co., Ltd., Japan) containing streptomycin (50 µg/ml) and rifampicin (25 µg/ml), and on those with additional antibiotics to which an R(+) strain was resistant. To assess the ratio between the numbers of R(+) and R(−) strains, 50 colonies on the streptomycin and rifampicin containing plates were subcultured on other plates containing appropriate drugs and their drug resistance was determined.

Results

The results are summarized in Table 2. When *E. coli* C SmRif was used as a host strain, both R(+) and R(−) strains decreased rapidly in fecal samples in the cases of R 144 and R 6 K. In the cases of RP 4 and pKO916 Km, the numbers of both R(+) and R(−) strains were well maintained until day 8, however, they decreased thereafter. Since *E. coli* C SmRif was derived from a laboratory strain of K-12 which was designed to be difficult to survive in the environment for molecular biological safety standards, those strains might be eliminated quickly from the intestine. In the case of each R plasmid, differences in number between R(+) and R(−) strains were not detectable, except that differences in the cases of R 6 K on day 1 and pKO 916 Km on day 14 were significant ($P < 0.05$, $F = 26.4$ and

Table 1. R plasmids used for colonization experiments

R plasmid	Incompatibility group	Resistance pattern*	Original host bacteria
RP4	Inc P	Tc, Km, Ap	<i>Pseudomonas aeruginosa</i>
R6K	Inc X	Tc, Km	<i>Proteus rettgeri</i>
R144	Inc Ia	Tc, Km	<i>Salmonella typhimurium</i>
pK0916Km	ND**	Km	<i>Escherichia coli</i>
pK0916Ap	ND	Ap	<i>E. coli</i>

* : Tc, tetracycline ; Km, kanamycin ; Ap, ampicillin

** : Not defined

Table 2. Changes in *E. coli* populations in fecal samples after inoculation

Host strain	R plasmid	Days after inoculation				
		1	2	4	8	14
C SmRif	RP4 (–)	8.1±0.4*	8.3±0.3	7.9±0.5	7.1±0.2	4.0±0.5
	RP4 (+)	8.5±0.4	8.7±0.2	7.9±0.3	7.5±0.3	4.3±0.7
	R144 (–)	6.4±0.4	6.0±0.4	4.4±0.7	<3.2**	<3.0
	R144 (+)	5.8±0.6	5.9±0.5	4.1±0.8	<3.4	<3.1
	R6K (–)	8.8±0.1	8.9±0.2	6.2±0.5	<3.0	<3.0
	R6K (+)	8.2±0.2	8.1±1.0	5.7±0.7	<3.0	<3.0
	pK0916Km (–)	8.1±0.4	8.3±0.4	7.9±0.3	7.2±0.4	3.9±0.1
	pK0916Km (+)	8.3±0.3	8.5±0.3	8.0±0.2	7.3±0.3	4.4±0.1
C25 SmRif	RP4 (–)	8.8±0.3	8.6±0.3	8.1±0.2	7.5±0.5	5.6±0.5
	RP4 (+)	8.6±0.4	8.6±0.2	7.5±0.1	5.7±0.4	<3.0
	R144 (–)	6.2±0.6	5.7±0.6	4.3±0.7	<3.0	3.7±0.6
	R144 (+)	5.1±0.2	4.0±0.5	3.2±0.3	<3.0	<3.0
	R6K (–)	9.2±0.2	9.3±0.1	8.5±0.3	5.4±0.8	4.7±1.2
	R6K (+)	8.4±0.2	8.5±0.4	7.1±0.4	3.7±0.4	<3.3
	pK0916Km (–)	9.0±0.2	9.2±0.2	8.7±0.1	7.4±0.1	4.8±0.4
	pK0916Km (+)	7.9±0.2	6.4±0.4	3.9±0.5	3.7±0.4	<3.0
cE916 SmRif	RP4 (–)	8.8±0.2	8.8±0.2	8.4±0.3	7.8±0.3	7.2±0.2
	RP4 (+)	8.5±0.3	8.4±0.2	7.8±0.4	6.8±0.2	5.3±0.3
	R144 (–)	8.9±0.4	9.0±0.3	8.8±0.1	8.2±0.2	7.8±0.5
	R144 (+)	8.4±0.4	8.0±0.5	7.4±0.2	5.9±0.3	4.5±0.4
	pK0916Ap (–)	8.9±0.3	8.9±0.2	8.4±0.2	8.2±0.2	7.5±0.3
	pK0916Ap (+)	8.9±0.4	9.0±0.2	8.4±0.1	7.8±0.2	6.8±0.3
	pK0916Km (–)	7.8±0.5	7.6±0.2	<7.1	6.4±0.3	<6.1
	pK0916Km (+)	9.0±0.5	9.0±0.2	8.4±0.2	7.4±0.3	7.8±0.2
E916 SmRif	pK0916 (+)	9.1±0.2	9.2±0.3	8.7±0.2	7.9±0.2	7.5±0.4
	pK0916Km (+)	8.5±0.2	8.1±0.4	7.4±0.3	5.9±0.6	5.7±0.5
	pK0916Ap (+)	8.2±0.5	6.9±0.3	5.0±0.7	<3.2	<3.1

* : Mean log₁₀ viable bacteria per gram of feces±standard deviation.
(n=5)

** : Viable number in some samples was below minimal detection level.

$P<0.01$, $F=73.6$, respectively).

When *E. coli* C 25 SmRif was used as a host strain, R(–) strains continued to predominate over the R(+) counterparts throughout the experimental period in all the cases of R plasmids. The numbers of organisms in fecal samples diminished gradually, except that of pK0916Km-bearing strain which decreased rapidly. Differences on day 4 in the case of each plasmid were significant (RP 4 : $P<0.01$, $F=31.3$; R 144 : $P<0.05$, $F=7.5$; R 6 K : $P<0.01$, $F=31.3$; pK0916Km : $P<0.01$, $F=345$).

On day 14, the numbers of R(–) strains reached the levels of $10^{3.7}$ to $10^{5.6}$ per gram of feces, whereas the mean numbers of R(+) strains were below the minimal detectable level.

When *E. coli* cE916 SmRif was employed as a host strain, the numbers of the *E. coli* strains were maintained well for 2 weeks. RP4- and R144-positive strains were inferior to their R(–) counterparts in number, however, in the case of pK0916Ap, the difference in number between R(+) and R(–) strains was smaller than that in the cases of RP 4 and R144 (on

day 14, RP4: $P < 0.01$, $F = 102$; R144: $P < 0.01$, $F = 131$; pKO916Ap: $P < 0.05$, $F = 11.0$). Furthermore, in the case of pKO916Km, the predominant strain in the fecal flora was the R(+) strain throughout the entire experimental period (on day 14, $P < 0.01$, $F = 100$).

Finally, the comparison study among *E. coli* strains which possessed different plasmids originated from the same cryptic plasmid, pKO916, was performed. *E. coli* E916 SmRif carrying the original pKO916, pKO916Km-positive E916 SmRif and pKO916 Ap-positive E916 SmRif were together given to streptomycin-treated mice and their viable numbers in fecal samples were examined. As shown in Table 2, the population of E916 SmRif was well maintained in the fecal flora for 14 days, whereas the numbers of pKO916Km-positive strain decreased gradually and the pKO916 Ap-positive strain was excluded rapidly from the intestinal flora (on day 4, pKO916 vs. pKO916 Km: $P < 0.01$, $F = 58.3$; pKO916 vs. pKO916 Ap: $P < 0.01$, $F = 102$; pKO916Km vs. pKO916 Ap: $P < 0.01$, $F = 40.0$). Eight days after inoculation, the viable numbers of E916 SmRif, pKO916Km-positive and pKO916 Ap-positive strains were $10^{7.9}$, $10^{5.9}$ and below $10^{3.2}$ per gram of feces, respectively.

Discussion

The viable numbers of R(+) strains of *E. coli* C SmRif, C25 SmRif and cE916 SmRif, except cE916 SmRif(pKO916Km), in fecal samples were similar to or lower than those of their R(-) counterparts during the entire experimental period, indicating that R plasmids used in this study did not have any positive effects on colonization of *E. coli* strains in the alimentary tract. Drug resistance patterns of R plasmids also appeared not to have any influence on bacterial colonization of the intestinal tract. Smith [14] and Anderson [1] described that it was difficult for *E. coli* strains possessing R plasmids to persist in the intestine without antibiotic pressure. In general, acquisition of R plasmids and probably other foreign DNAs seems to be a burden on the organisms in the struggle for existence in the normal intestinal flora [2].

Although the ability of organisms possessing R plasmids to colonize in the alimentary tract is affected by various factors such as

bacterial serotypes [5, 10] and ages of the animal [8, 9], the relation between the host organisms and plasmid DNAs may also be important, since the majority of drug-sensitive strains of *E. coli* that were isolated from feces of healthy laboratory animals with standard selective media containing no antibiotics, and therefore, form a major component of the intestinal flora, carry plural cryptic plasmids, some of which seem to be candidates for R plasmids [11, 12]. In contrast with the foreign plasmid DNAs the cryptic plasmids existing in the drug-sensitive strains appeared to cooperate with their own host organisms to outlive the struggle for existence in the intestinal flora. Our results indicated that possession of the modified cryptic plasmid, pKO916Km, could complement the reduced colonizing ability of *E. coli* cE916 SmRif that lacked the intact cryptic plasmid, pKO916. In comparison to pKO916, however, pKO916Km, in which a kanamycin-resistance transposon (Tn5) was located, could not recover the perfect competence of the host strain to persist in the fecal flora, and pKO916Ap containing an ampicillin-resistance transposon (Tn2602) appeared to reduce it. Transposons are also foreign DNAs for host organisms and may have introduced mutation near the integration site. Since the favorable relationship between host organisms and cryptic plasmid DNAs must have been established during long period in the process of evolution, the possession of a converted R plasmid by the original host strain of *E. coli* may minimize the decline of the ability to colonize in the alimentary tract.

The mechanism by which pKO916-carrying E916 strain was well colonized in the alimentary tract in contrast with other R(+) strains are still unknown. Many enterotoxigenic *E. coli* strains possess colonization factor antigens (CFA) associated with different serologically typable pili, whose expression requires plasmid- and/or chromosome-encoded genes [3]. A few nonpathogenic *E. coli* strains, which were isolated from feces of a healthy human, were reported that their attachment to the intestinal walls is mediated by plasmid DNAs, however, putative colonization genes present on the plasmids have not been cloned out yet [4, 15]. We don't have any evidence which indicates that the differences among strains in E916 to persist in the intestine correlated to their bind-

ing ability to mucosal cells. Our results, however, suggested that an ampicillin-resistance transposon (Tn2602) was located within the critical region for expression of the gene that encoded the colonizing ability. Cloning out the gene corresponding to the colonization phenotype through the use of the transposon for probe and characterization of the mechanisms to persist in the intestine will provide more information to understand the epidemiology of plasmid DNAs and the constitution of the intestinal flora.

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Rプラスミド保有大腸菌のマウス腸管内定着性

下田耕治・久原孝俊*・前島一淑

慶應義塾大学医学部

*帝京大学医学部

Rプラスミド保有大腸菌のマウス腸管内定着性について検討するため、Rプラスミド保有菌および非保有対照菌を同時にストレプトマイシン処理マウスに経口投与し、経時的に糞便内菌数を測定した。その結果、Rプラスミド保有菌の菌数は非保有菌に比べ同じか低値であっ

たが、本来宿主菌が保有していた *cryptic* プラスミドに由来するRプラスミドでは逆に保有菌の方が非保有菌よりも多く検出された。以上の成績から宿主菌対Rプラスミドの関係は宿主菌の腸管内定着性に影響を及ぼすことが示唆された。