Conversion of Cryptic Plasmids into R Plasmids in Drug Sensitive
Escherichia coli Isolated from Laboratory Mice and Rats

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Of 21 drug sensitive Escherichia coli strains isolated from laboratory mice and rats, 17 harbored 1 to 9
cryptic plasmids. To examine the convertibility of these cryptic plasmids into conjugative R plasmids, we
introduced a nonconjugative plasmid, pMK1::Tn2602 (ColE1::Tn5::Tn2602), which encodes resistance to
kanamycin (Km) and ampicillin (Ap), into a mouse strain E916 possessing at least 5 plasmids by
transformation. A mixed culture of the transformant E 916 (pMK1::Tn2602) as donor and E. coli K-12
strain as the recipient produced Km-resistant and Ap-resistant transconjugants at the frequencies of 10^{-9}
and 10^{-6} per donor cell, respectively. Agarose gel electrophoretic analysis revealed that the Km-resistant
transconjugant had a single plasmid (pKO916Km) of 29.5 megadaltons (Mdal), which was capable of
transforming another strain of E. coli K-12 into a Km-resistant. Similarly, the Ap-resistant transconju-
gant had a single conjugative R plasmid (pKO916Ap) of 29 Mdal. Restriction enzyme cleavage and
electron microscopic analysis demonstrated that these R plasmids were the composite plasmids consisting
of a 26 Mdal cryptic plasmid presented in the strain E916 and either of a Km transposon (Tn5) or an Ap
transposon (Tn2602). They were conjughally transmissible to different species of enterobacteria such as
Shigella and Salmonella. In vivo transfer of pKO916Ap was also demonstrated in gnotobiotic mice.—Key
words: Escherichia coli, Plasmid, Mouse, Rat.

The isolation frequency of drug resistant organisms from laboratory animals is much lower than that from humans or domestic
animals because of the principle of avoiding the use of antibiotic agents in animals for research. However, routine administration of
antibiotics has resulted in an increase in drug resistant organisms and R plasmids in colonies
of laboratory mice and rats [5, 12]. This situation could be similar to the first stage of appearance of drug resistant organisms in
man and domestic animals. We therefore attempted to isolate plasmids from Escherichia coli of laboratory mice and rats, and
found that various cryptic plasmids were detectable in many drug sensitive organisms. This paper deals with the possible convert-
ibility of such cryptic plasmids into conjugative R plasmids through drug resistance trans-
posions.

MATERIALS AND METHODS
Bacterial strains and plasmids: Twenty-one drug sensitive E. coli were isolated from
14 laboratory mice and 7 rats of 15 breeding colonies. The mouse strain E916, containing
at least 5 plasmids, was used in this experiment. E. coli ML1410 (naldixic acid-resistant,
methionine-requiring F-derivative of K-12) and the rifampicin-resistant derivatives of
other genera of bacteria listed in Table I were used as the recipient in mating experiments. For in vivo mating, E. coli C (rifampicin-re-
sistant mutant) containing an ampicillin (Ap) resistance plasmid (pKO916Ap) was employ-
ed as donor, and ML1410 and endogenous fecal E. coli strain (Fig. 1, lane C) were used
as the recipient. A nonconjugative plasmid,
pMK1::Tn2602, which consisted of a ColE1 molecule and dual transposons, Tn5 and Tn-2602, resistant to kanamycin (Km) and Ap, respectively, was kindly provided by T. Yamamoto [14].

Isolation of plasmid DNA: Plasmid DNAs were isolated by CsCl-ethidium bromide gradient centrifugation as described previously [7]. Samples were centrifuged in a Vti65 rotor in a Bekman L5-50B ultracentrifuge at 37,000 rpm for 12 h at 20°C.

Transformation and conjugation: Transformation with plasmid DNA was performed by the method of Cohen et al. [2] using a drug sensitive E. coli E916 as the recipient. Conjugal transfer of drug resistance was examined by mixed cultivation for 1 h at 37°C [13].

Agarose gel electrophoresis: Gel electrophoresis was carried out by the method of Meyers et al. [6]. The procedures for preparation of the gels and electrophoresis have been described [7], except that 0.8% agarose 1600 (Waco Pure Chemical Industries, Ltd.) was used. Molecular weight determinations were based on the electrophoretic mobilities using pTE500 [11], pMK1::Tn2602 [14], and lambda phage DNA digests.

Recovery of cryptic plasmids from agarose gel: Cryptic plasmids isolated from E916 were applied to a 0.8% agarose gel and electrophoresed at 100 V for 4 h. Each fraction of cryptic plasmids was cut out from the stained gel, and then packed into visking cellulose tubing with a small amount of running buffer. After electrophoresis of the tubing at 4°C overnight, the plasmid DNA in the eluate from the agarose gel was extracted with saturated phenol and precipitated with cold ethanol in 300 mM sodium acetate.

Restriction endonuclease digestion: The endonucleases EcoRI, BamHI and HindIII were purchased from Takara Shuzo Co., Ltd. All DNA digestions were carried out at 37°C for 1 h in 50 μl of the reaction mixture. The buffers used were as follows: for EcoRI, 100 mM Tris-HCl (pH 7.5), 50 mM NaCl and 10 mM MgCl₂; for BamHI, 100 mM Tris-HCl (pH 7.5), 10 mM MgCl₂ and 6 mM 2-mercaptoethanol (2-ME); and for HindIII, 6 mM Tris-HCl (pH 7.5), 60 mM NaCl, 6 mM MgCl₂ and 6 mM 2-ME.

Electron microscopic analysis of self-annealed molecules: Following the method of Sekizaki et al. [11], DNA samples were denatured with 0.25 N NaOH, and then neutralized with 1M Trizma-HCl (Sigma). Dialyzed DNA samples in 50% formamide were spread on a 18% hypophase, picked up on grids, stained with uranyl acetate, and shadowed with platinum-palladium (80:20).

In vivo transfer of pKO916Ap: Female germ-free ICR mice were obtained commercially from CLEA Japan Inc. Eight mice were used in each of the experiments. The mice were kept in isolators sterilized with 2% peracetic acid and fed on commercial pellets irradiated with 5 Mrad of gamma-rays (Funa-bashi Farm). They received 0.1 ml of bacterial culture or 10% mouse fecal mixture of 10 Sdc: ddY mice via a stomach tube. Feces were collected from each of the mice and plated on DHL agar (Eiken Chemical Co., Ltd.) containing relevant drugs after serial dilutions. About 100 colonies on agar plates were picked up and determined for their drug resistance.

RESULTS

Cryptic plasmids in drug sensitive E. coli: Of the 21 drug sensitive E. coli strains isolated from laboratory mice and rats, 17 possessed multiple plasmids species. The numbers of plasmids per strain ranged from 1 to 9, with an average of about 5. Plasmid DNAs were detected in 12 out of the 14 mouse strains and 5 out of the 7 rat strains. Analytical gel electrophoresis revealed that the patterns of cryptic plasmids, except for lanes C and H, were different among the strains isolated from laboratory mice and rats (Fig. 1). However, 2 strains from mice that had an identical origin and had been separated 6
months before the present investigation, exhibited the same migration pattern (Fig. 1, lanes C and H). Cryptic plasmids might be well maintained in the organisms of laboratory animals. These plasmids were between approximately 3 megadaltons (Mdal) and 105 Mdal in size.

Convertibility of cryptic plasmids into R plasmids: One of the drug sensitive strains, E916, carrying at least 5 cryptic plasmids (Fig. 1, lane E) was transformed with a non-conjugative plasmid, pMK1::Tn2602 (ColE-1::Tn5::Tn2602) encoding Km and Ap resistance. A mixed culture of the transformant E916(pMK1::Tn2602) and E. coli ML1410 produced Km-resistant transconjugant and Ap resistant transconjugant ML1410 at frequencies of $10^{-9}$ and $10^{-6}$ per donor cell, respectively. Each of these transconjugants contained a single conjugative R plasmid, designated pKO916Km and pKO916Ap, estimated at 29.5 Mdal and 29 Mdal, respectively, in size (Fig. 2). Since the molecular weights of Tn5 and Tn2602 were 3.5 Mdal and 3 Mdal, respectively [14, 15], these R plasmids appeared to have originated from a single cryptic plasmid (pKO916) slightly smaller than themselves (Fig. 2, arrow). To confirm this hypothesis, we purified the cryptic plasmid pKO916 from a preparative agarose gel, and digested pKO916Km, pKO916Ap and pKO916 with restriction endonucleases of EcoRI, BamHI and HindIII (Fig. 3). The EcoRI and BamHI cleavage patterns were difficult to discriminate between pKO916Km and pKO916Ap. It appeared that Tn5 and Tn2602 were independently translocated on an identical cryptic plasmid, since both transposons had no cutting site for EcoRI and a single site for BamHI within their nucleotide sequences [14, 15]. HindIII digestion of pKO916 and pKO916Ap produced 2 similar cleavage fragments (Fig. 3, lanes H and C) and that of pKO916Km produced 4 fragments (Fig. 3, lane B). Our hypothesis was supported by these observa-

![Fig. 1. Agarose gel electrophoresis of cryptic plasmids isolated from 13 drug sensitive E. coli strains. Lanes D, I and L are of rat origin, and other lanes are of mouse origin. Numbers indicate the size (in mega-daltons), and the arrow shows the region of chromosomal DNA fragments.](image1)

![Fig. 2. Agarose gel electrophoreses of pKO916Km and pKO916Ap. Lanes: (A) cryptic plasmids of E916 (Fig. 1, lane E), (B) pKO916Ap, (C) pKO916Km, (D) standard plasmid DNAs of pTE500 (64 Mdal) and pMK1::Tn2602 (10.7 Mdal). The arrow indicates an original cryptic plasmid of pKO916Km and pKO916Ap in E916.](image2)
tions, since Tn5 carried 2 recognition sites for HindIII, whereas Tn2602 carried no site for HindIII [14, 15]. Moreover, electron microscopic analysis of self-annealed DNAs revealed that Tn5 was located on pKO916Km with a peculiar stem-loop conformation, and Tn2602 with a short inverted repeat structure was located on pKO916Ap (Fig. 4). The molecular weight of pKO916 was estimated to be 26 Mdal. These results strongly suggested that pKO916 was the original transferable cryptic plasmid in E916 and was converted into R plasmid containing either Tn5 or Tn-2602. These R plasmids were transferable to E. coli, Shigella, Citrobacter, Salmonella, Klebsiella, Enterobacter, Serratia and Yersinia by conjugation at frequencies of $10^{-3}$

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**Fig. 4.** Electron micrographs of self-annealed pKO916Km (A) and pKO916Ap (B). IR, Inverted repeat structure of double-stranded DNA. Bars, 1 μm.

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**In vivo transfer of pKO916Ap:** The results of transfer of pKO916Ap in the intestine of mice are summarized in Fig. 5. In gnotobiotic mice inoculated with recipient strain
Table 1. Transfer of pKO916Km and pKO916Ap to various bacteria

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Transfer frequency$^a$ of</th>
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<tbody>
<tr>
<td></td>
<td>pKO916Km</td>
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<tr>
<td><em>Escherichia coli</em></td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td><em>Sh. sonnei</em></td>
<td>$10^{-5}$</td>
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<tr>
<td><em>Citrobacter freundii</em></td>
<td>$10^{-4}$</td>
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<tr>
<td><em>Salmonella typhimurium</em></td>
<td>$10^{-4}$</td>
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<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>$10^{-4}$</td>
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<tr>
<td><em>Enterobacter cloacae</em></td>
<td>$10^{-5}$</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>$10^{-4}$</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>$10^{-6}$</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>$&lt;10^{-8}$</td>
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<tr>
<td><em>P. vulgaris</em></td>
<td>$&lt;10^{-8}$</td>
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<tr>
<td><em>P. rettgeri</em></td>
<td>$&lt;10^{-8}$</td>
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<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>$&lt;10^{-8}$</td>
</tr>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td>$&lt;10^{-8}$</td>
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$^a$ Expressed as the number of transconjugants per that of donors after mixed culture at 37°C for 1 h.

DISCUSSION

The majority of *E. coli* strains isolated from laboratory animals were found to be drug sensitive [12]; however, over 80% of them possessed various cryptic plasmids. Our results indicated that one of these cryptic plasmids, pKO916, was self-transferable among different bacteria, and was converted to R plasmids by the translocation of drug resistance transposons. These facts suggested that conjugal plasmids present in drug susceptible organisms could be evolved to R plasmids through the acquisition of drug resistance from transposons on exposure to antibiotic pressure [1, 8, 9].

Transfer of R plasmid in the intestine of mice inoculated with both donor and recipient has been examined by Duval-Ilfah et al. [3], Kasuya [4] and Salzman and Klemm [10]. They observed that the rate of in vivo transfer was much slower than that in vitro. However, the results of the present investigation indicated that the transfer of R plasmid was very fast without the selecting pressure of antibiotic treatment. This discrepancy could depend on the specific mating pair and the kind of R plasmids, since *Serratia liquefaciens* [3], *Shigella flexneri* [4], and *Klebsiella pneumoniae* [10] were used as the donor strain, and multiple drug resistance plasmids were employed in the previous reports.

Although application of antibiotic agents for laboratory animals is restricted, the prophylactic use of antibiotics is tolerated in a few laboratory animal colonies of breeders and testing laboratories in Japan [5, 12]. If antibiotics were widely applied to laboratory animals, drug resistant organisms and R plasmids would tend to spread and become extensively distributed in this field. The results obtained here demonstrated that R plasmid being well transferred in gnotobiotic mice could not distribute easily in the digestive tract associated with the whole fecal flora in the absence of antibiotic selecting pressure.
Therefore, to prevent an increase of drug resistant organisms and R plasmids, we must avoid the easy administration of antibiotic agents to laboratory animals for the prevention of bacterial diseases. Control of infectious diseases in laboratory animal colonies should be made by other means such as the establishment of a microbiological barrier system.

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REFERENCES

CONVERSION OF CRYPTIC PLASMIDS TO R PLASMIDS


要約
実験動物由来薬剤感受性大腸菌に存在する Cryptic プラスミドの R プラスミド化について：下田耕治、前島一世、関崎 雄1、寺門誠已1（慶應義塾大学医学部実験動物センター、1農林水産省家畜衛生試験場）実験動物由来薬剤感受性大腸菌21株の中7株は1〜9栄の cryptic プラスミドを有していた。これらの cryptic プラスミドが R プラスミド化するかどうか確めるため、非伝達性プラスミド pMK1:: Tn2602 (ColEl:: Tn5:: Tn2602, Km・Ap 耐性) を複数の cryptic プラスミドを有するマウス由来株 E916 へ導入して得られた transformant E916(pMK1:: Tn2602) を供与菌として、大腸菌 K-12 株を受容菌として接合伝達試験を行った。Km 耐性 transconjugant および Ap 耐性 transconjugant がそれぞれ 10−9 および 10−8 の頻度で分離された。Km 耐性 transconjugant から分子量 29.5 Mdalの伝達性 R プラスミドが、Ap 耐性 transconjugant から 29 Mdal の伝達性 R プラスミドが検出された。DNA 解析の結果、これらの R プラスミドは、E916 に存在する 26 Mdal の cryptic プラスミドと Km transposon (Tn5) あるいは Ap transposon (Tn2602) のどちらか一方からなるプラスミドであることがわかった。これらは他の腸内細菌科の菌へ伝達可能であり、また in vivo の伝達も観察された。