Plasmid-Determined Epistatic Susceptibility to Kasugamycin

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The effect of two representative plasmids, R100 and F8-gal, on the susceptibility of Escherichia coli to kasugamycin was studied. R+ and F+ cells were found to be more susceptible to this antibiotic than R− and F− cells, respectively. Retransfer and curing experiments of these plasmids show that this increased susceptibility of host cells to kasugamycin was conferred by either of the plasmids. At the early stage of growth of R100+ cells in the presence of kasugamycin, R− segregants overgrew the population and then they were replaced by kasugamycin-resistant mutants of the R+ cells which became the majority cell line of the population. The former phenomenon is assumed to be due to the increased susceptibility of R100+ cells to kasugamycin, and the latter is probably related to the finding that R100 enhances the spontaneous mutation of host cells to resistance to kasugamycin. The practical and experimental significance of these findings are discussed.

R plasmids were originally characterized as conjugatively transmissible genetic factors determining resistance to various drugs (1, 9). However, the notion implicit in the so-called R(resistance) plasmids has been gradually modified by extensive studies in this field which have revealed that plasmids may also increase the susceptibility rather than the resistance of the host cells to various chemicals, inorganic and organic. The examples are the acridine dyes (17) including atabrine (18), sodium dodecyl sulfate (5, 13), nalidixic acid (16), macarboxymycin (7), and moenomycin (15).

Kasugamycin (Ksg), an aminoglycoside antibiotic which has been isolated from Streptomyces kasugaensis, is a selective inhibitor against bacterial protein synthesis (12, 14). This report presents evidence that plasmids R100 and F8-gal confer an increased susceptibility to this antibiotic upon the host cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. Several substrains of Escherichia coli K-12 were used. They were CSH2 (F−, met, pro), YC72 (F+, thi, thr, leu, thy, lac), JE346 (F−, pur, trp, gal, lac, xyl, ara, mtl, str, pho, tss, lamin+), and W4570 (F−, gal, lac, mal, xyl, ara, mtl, str). From these strains, various derivatives (Table 1) were prepared to test Ksg susceptibility. R100 (f−, FII incompatibility group, sul, str, cml, tet, the gene markers, mtl, str, cml, and tet coding for resistance to sulfonamides, streptomycin, chloramphenicol, and tetracycline, respectively), R100-1 (a fertility derepressed derivative of R100) (2), and F8-gal (4) were used as plasmids.

Media and drugs. Penassay broth (PA broth, Difco), supplemented with 10 µg of thymine per ml, was used as the liquid medium, and eosin methylene blue (EMB)-lactose agar (EMB-lac, Eiken, Tokyo), EMB-agar without sugar (an order-made product by Kyokuto, Tokyo), and synthetic EMB (EM)-sugar agar (3, 6) were used as the solid media. EMB-agar contains the same components as EMB-lac except for lactose and was used by adding appropriate sugar at 1%. EM-sugar agar was a synthetic salts-sugar agar containing the sugar as the sole carbon source. Ksg sulfate (844-µg equivalent per mg) was a kind gift of H. Umezawa, Institute of Microbial Chemistry, Tokyo. The sodium salt of penicillin G (Banyu, Tokyo) and chloramphenicol (CM; Sankyo, Tokyo) were purchased commercially.

Susceptibility test by measuring bacterial growth. Two different methods were employed. (i) Cells with and without a plasmid were grown in PA broth to give an absorbance of 0.7 at 600 nm with a Schimadzu-Bausch and Lomb spectrometer, model 20. A portion of 0.05 ml of these cultures was inoculated into 5 ml of PA broth containing various concentrations of Ksg and incubated at 37 C without shaking. The growth curve was followed turbidimetrically by reading the absorbance at 600 nm. (ii) For viable cell counts, samples were appropriately diluted with unbuffered physiological saline and 0.1-ml dilutions were plated on EMB-lac. After 24 h of incubation at 37 C the colonies were counted.

Resistance distribution curve to Ksg. Less than 104 viable cells from a fresh culture were inoculated into PA broth, grown overnight at 37 C, and then plated after appropriate dilution on EMB-sugar containing varying concentrations of Ksg. The number of colonies formed after 48 h at 37 C was counted and a resistance distribution curve to Ksg for isogenic strains with and without a plasmid was made.
**Conjugative transfer of the plasmid.** Donor (0.1 ml) and 0.9 ml of the recipient in the exponentially growing phase were added to 1.0 ml of fresh PA broth. After 60 min of incubation at 37°C with gentle shaking, the mixture was washed, diluted with saline, and then plated on EM-sugar agar selective for the transconjugants. After 72 h of incubation at 37°C, colonies were picked, purified, and confirmed for their genetic markers.

**Methods for plasmid curing.** In view of a possibility that curing might be due to epistatic susceptibility to the agent used for it (17), no curing agent was used, but the penicillin screening method was employed to obtain spontaneous R⁻ segregants from R⁺ cells. Penicillin G (400 μg) and 25 μg of CM per ml were added to exponentially growing R⁺ cultures in PA broth and incubated at 37°C with gentle shaking for 6 h. Then the cells were washed with saline, appropriately diluted, and plated on EMB-lac which was incubated at 37°C for 48 h. One hundred to 200 colonies formed were picked and restreaked on EMB-lac plates. After incubation for 24 h their drug resistance was examined by replica plating on EMB-lac containing CM at 25 μg per ml. Several CM-susceptible colonies thus obtained were picked from the master plates, purified, and examined for their resistance markers other than that to CM, and if shown to be susceptible they were used as R⁻ segregants. Selection by f2 phage was applied to obtain an F⁻ segregant from F⁺ cells. Ar overnight culture of F8-gal⁺ strain was mixed with f2 phage, and CaCl₂ was added at a concentration of 2 mM and then incubated for 48 h at 37°C. Appropriate dilutions of this mixture were plated on EMB-gal. After 72 h of incubation at 37°C, galactose-nonfermenting colonies were picked and purified, their genetic characteristics were confirmed, and then they were used as F⁻ segregants.

**RESULTS**

**Growth of R⁻ and R⁺ cells in Ksg.** Typical growth curves of CSH2 with and without R100 are shown in Fig. 1. Ksg initially inhibited the growth of R⁻, CSH2, as well as R⁺ bacteria, YC190 [= CSH2(R100)], at 640 μg per ml. The growth inhibitory period of this drug was longer with R⁺ bacteria than R⁻ at concentrations higher than 160 μg of Ksg per ml. In the absence of Ksg, no difference was observed in the growth between the R⁺ and the R⁻ cells. This suggests that the R plasmid confers an increased susceptibility to Ksg upon its host bacteria. A retransfer experiment with the same R plasmid was performed. R100 was retransferred from YC190 [= CSH2(R100)] to two strains, YC72 and JE346. Although the R⁻ strains, YC72 and JE346, grow in the presence of 320 and 1,280 μg of Ksg per ml, respectively, their R⁺ derivatives, YC196 [= YC72(R100)] and YC199 [= JE346(R100)], also show increased susceptibility to it and could not grow within 48 h of incubation (data not shown). Figure 2 shows the effect of curing. The strain YC190 [= CSH2(R100)] was subjected to penicillin screening and an R⁻ segregant was obtained. This R⁻ segregant, YC192 [= CSH2(R100)⁻ pen], was more resistant to Ksg than an R⁺ strain, YC191

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**Table 1. Strains used**

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<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Plasmids</th>
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<tbody>
<tr>
<td>CSH2</td>
<td>As CSH2 but R⁺</td>
<td>R100</td>
</tr>
<tr>
<td>YC190</td>
<td>As YC190 but treated with penicillin</td>
<td>R100</td>
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<tr>
<td>YC191</td>
<td>As YC191 but cured of R by penicillin selection</td>
<td>R100</td>
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<tr>
<td>YC192</td>
<td>As YC190 but resistant to Ksg</td>
<td>R100</td>
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<tr>
<td>YC193</td>
<td>As YC193 but treated with penicillin</td>
<td>R100</td>
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<tr>
<td>YC194</td>
<td>As YC194 but cured of R by penicillin selection</td>
<td>R100</td>
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<tr>
<td>YC195</td>
<td>As YC195 but treated with penicillin</td>
<td></td>
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<tr>
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<td>As YC72 but R⁻ by transfer from YC190</td>
<td>R100</td>
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<tr>
<td>YC196</td>
<td>As YC196 but R⁻ by transfer from YC193</td>
<td>R100</td>
</tr>
<tr>
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<td>As YC72 but R⁻</td>
<td>R100-1</td>
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</tr>
<tr>
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<td>As JE346 but R⁺ by transfer from YC190</td>
<td>R100</td>
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<td>As JE346 but R⁺ by transfer from YC190</td>
<td>F8-gal</td>
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<td>As YC200 but F⁻</td>
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<td>As YC200 but treated with f2 phage</td>
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<td>As W4573 but F⁺ by transfer from YC200</td>
<td>F8-gal</td>
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<tr>
<td>YC203</td>
<td>As W4573 but F⁺ by transfer from YC200</td>
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*Methods of derivation were as described."
obtained for R100 with F8-gal,

the growth as experiments strain, JE346 later increase the as same the of ug YC192 [= W4573 (F8-gal)] recovered the original resistance to Ksg of the F- parent (Fig. 5) in the presence of 320 μg of Ksg per ml. Contrary to YC202 [= JE346(F8-gal-)f2], YC201 [= JE346(F8-gal+)f2], an F+ strain which had escaped from this selection, retained susceptibility to Ksg and its growth was inhibited markedly by 320 μg of Ksg per ml.

Appearance of Ksg-resistant mutants in R+ cultures. CSH2 and YC190 [= CSH2(R100)] were grown in the presence of varying concentrations of Ksg. When 640 μg of Ksg per ml was added in a liquid medium, YC190 [= CSH2(R100)] was almost completely inhibited by Ksg as shown in Fig. 1. However, YC190 [= CSH2(R100)] in Ksg at a concentration lower than 320 μg per ml showed interesting growth curves. The turbidity of the YC190 [= CSH2(R100)] in the presence of 160 μg of Ksg per ml increased slightly earlier than that of the R+ parent, it remained constant during the

Fig. 1. Effect of Ksg on the growth of R- and R+ strains. CSH2 (O) and YC190 [= CSH2(R100)] (●) were incubated in PA broth with Ksg at concentration of 640, 320, 160, and 80 μg/ml, and no addition (control). OD, Optical density.

which had experienced the same treatment as YC192 [= CSH2(R100-) pen], but which had escaped from the selective killing by the penicillin screening. The penicillin screening resulted in a greater than usual susceptibility. This is the reason why we used YC192 [= CSH2(R100-)pen] rather than CSH2 as the R- control and YC191 [= CSH2(R100+) pen] instead of YC190 [= CSH2(R100)] as the R+ control.

Growth of F- and F+ cells in Ksg. Similar experiments as described in the previous section for R100 were performed with the F plasmid. The growth of an F+ strain, YC200 [= JE346(F8-gal)], obtained by infecting JE346 with F8-gal, was inhibited in the presence of 320 μg of Ksg per ml and the turbidity started to increase later than that of the parental F- strain, JE346 (Fig. 3). Essentially the same

results as those of JE346 and YC200 [= JE346 (F-8gal)] were obtained with W4573 and YC203 [= W4573 (F8-gal)] (Fig. 4). An F- segregant, YC202 [= JE346(F8-gal-)f2], obtained by f2 phage resistance selection from YC200 [= JE346(F8-gal)], recovered the original resistance to Ksg of the F- parent (Fig. 5) in the presence of 320 μg of Ksg per ml. Contrary to YC202 [= JE346(F8-gal-)f2], YC201 [= JE346(F8-gal+)f2], an F+ strain which had escaped from this selection, retained susceptibility to Ksg and its growth was inhibited markedly by 320 μg of Ksg per ml.

Fig. 2. Effect of Ksg on the growth of a cured strain. The R- strain YC192 [= SH2(R100-)pen] (O, dotted line) and an R+ strain YC191 [= CSH2(R100+) pen] (●, dotted line) isolated after penicillin screening of YC190 [= CSH2(R100)], with and without Ksg at 160 μg/ml. Results on CSH2 (O, solid line) and YC190 [= CSH2(R100)] (●, solid line) were also included for comparison. OD, Optical density.
subsequent 12-h incubation, and rapidly increased to attain a higher optical density than CSH2. To analyze this observation more precisely, the viable cell counts and the frequency of R- segregants in the YC190 (= CSH2(R100)) culture were measured during the growth in Ksg. As shown in Fig. 6, the viable counts of YC190 (= CSH2(R100)) decreased for about 12 h after an initial slight increase, then increased rapidly, and finally reached a count higher than that of CSH2. R- segregants appeared immediately in YC190 (= CSH2(R100)) cultures soon after beginning incubation in Ksg. They showed an initial increase and then later decreased with the increase of the turbidity and the viable counts. The resistance distribution curve of the CSH2 and YC190 (= CSH2(R100)) cultures in different concentrations of Ksg was shown in Fig. 7. YC190 (= CSH2(R100)) showed two different phases within a range of 0 to 160 μg of Ksg per ml on the solid medium, that is, a more and a less phase in the number of viable colonies in comparison to that of CSH2.

**Growth of Ksg-resistant mutant.** Several R+ colonies obtained from the YC190 (= CSH2(R100)) culture which had been incubated for 20 h in the presence of 160 μg of Ksg per ml were purified by successive single colony
isolations, serially subcultured five times without Ksg, and again grown in the presence of 160 μg of Ksg per ml. A typical growth curve in response to Ksg is shown in Fig. 8. This Ksg-exposed R+ strain, YC193 [CSH2(R100)ksg], grew more rapidly than the original R+ strain, YC190 [CSH2(R100)], in the presence of Ksg. No difference was observed in growth of these two strains in the absence of the drug. Thus, the Ksg-exposed R+ strain, YC193 [CSH2(R100) ksg], seems to be a Ksg-resistant mutant. To decide whether the determinant for Ksg resistance was located on the host chromosome or on the R plasmid itself, an R- segregant, YC195 [CSH2(R100-)]ksg pen], was obtained from the Ksg-resistant mutation strain, YC193 [CSH2(R100)ksg].
Csh2(R100)ksg], by penicillin screening and Ksg resistance was examined. This was still resistant to 160 μg of Ksg per ml to the same level as an R+ Ksg-resistant mutant, YC194 [= Csh2(R100+)ksg pen], which had experienced the same treatment as YC195 [= Csh2(R100-)ksg pen], but which had escaped from the selective killing by the penicillin (Fig. 9). On the other hand, the R plasmid of the R+ Ksg-resistant mutant, YC193 [= Csh2(R100)ksg], was retransferred to YC72 and the resulting R100+ strain of YC72, YC198 [= YC72(R100)ksg], was examined for its Ksg resistance. The R+ transconjugant, YC198 [= YC72(R100)ksg], did not inherit the Ksg resistance from the strain, YC193 [= Csh2(R100)ksg], as shown by a finding that the growth of YC198 [= YC72(R100)-ksg] was inhibited to the same level as the original R100+ strain, YC196 [= YC72(R100)], in the presence of 160 μg of Ksg per ml (Fig. 10). These results reveal that the determinant for the acquired Ksg resistance of YC193 [= CSH2(R100)ksg] isolated from R+ culture of YC190 [= CSH2(R100)] is not located on the R plasmid but on the host chromosome.

**DISCUSSION**

In this report it was demonstrated that two plasmids, R100 and F8-gal, adopted as representative plasmids, conferred epistatic susceptibility to Ksg upon the host *E. coli*. This susceptibility is an expression of the plasmid because susceptibility could be transferred by conjugal transfer of the plasmid, and it also disappeared after elimination of the plasmid from the cells. We used the word “epistatic” here to differentiate this type of susceptibility from mutations of resistance genes to susceptibility. The word “epistasis” may be used for any characters conferred by a plasmid which exerts a dominant effect to other possibly related characters on the chromosome.

These plasmids, on the other hand, seemed to increase the frequency of chromosomal mutation to Ksg resistance as shown by final higher
turbidity and viable counts of the plasmid-bearing strains compared to the nonbearers, and also by the higher frequency of appearance of Ksg-resistant mutants from the plasmid-bearing strains than from the nonbearers on agar plates containing Ksg at a concentration higher than one markedly affected by the epistatic susceptibility effect. The difference between R+ and R− cells in optical density and viable cell counts finally attainable in liquid medium as well as in the number of colonies formed on agar containing Ksg at concentrations of 120 μg or more per ml (Fig. 7) was quantitatively small but qualitatively reproducible. This was commonly observed in all drugs so far known to be epistatically susceptible.

The mechanism of epistatic susceptibility has been to some extent clarified for sodium dodecyl sulfate (5, 13), macaromycin (7), and monomycin (15). The killing effect of these drugs was shown to be more active when the regulatory mechanism for the conjugal fertility of the plasmid is derepressed, and hence the epistatic susceptibility was ascribed to the existence of pili (5, 7, 13, 15). There was no difference in susceptibility to Ksg between a strain carrying R100 and another carrying its fertility-derepressed mutant, R100-1. We cannot ascribe, therefore, the mechanism of this epistatic Ksg susceptibility to the existence of pili. The resistance mutation in plasmid-bearing strains to nalidixic acid (16) and Ksg was shown to be due to chromosomal mutation. But nalidixic acid was shown not to increase the mutation rates to chromosomal genes other than that of nalidixic acid resistance itself in strains carrying R100. An increased detrimental effect of nalidixic acid or Ksg to the host growth through increased permeability, for example, could be the mechanism for the epistatic susceptibility effect but not for the effect on the increased resistance mutation. It has been shown that F and R plasmids affect the protein-synthesizing machinery of the host cells. For example, Onishi reported the accelerated turnover of ribosomal RNA in F+ strains due to the presence of F plasmid (10). Another example is the inhibition of the propagation of T3 and T7 phages by F plasmid proposed to operate at the translational level (8, 11). These observations may be related to the epistatic susceptibility to Ksg of plasmid-bearing strains, possibly due to a similar effect of the plasmid on the protein-synthesizing mechanism, which in the presence of Ksg enhances the inhibitory effect of the antibiotic on protein synthesis.

Apart from the mechanism of these phenomena, we would point out the practical significance of these observations. We have been impressed by the relative ease of finding a drug to which the R plasmid-carriers are epistatically susceptible. The additional observation on increased mutation rate to resistance to the same drug might become an obstacle in practical use, but we believe a rational application of these observations might lead to valuable therapeutic agents and procedures. Ksg is also shown to be useful in obtaining a cured strain from a plasmid-carrying strain.

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LITERATURE CITED


