Distribution in Nature of R factors that Increase Susceptibility to Rifampin of rif-r Mutants in Escherichia coli

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Among 117 gram-negative bacteria isolated from pathological materials, 46 were found to carry antibiotic resistances transferable to Escherichia coli K-12; we therefore concluded that they carry infectious R factors. When transferred to a type of rifampin-resistant mutant of E. coli, all these R factors decreased the resistance to rifampin, but only 10% of them lowered the resistance to one-tenth or less that of the isogenic R⁻ strain. The relevance of these facts for the epidemiology of R factors in gram-negative bacteria is discussed.

In a previous paper we reported the existence of a class of rifampin-resistant mutants of Escherichia coli K-12 that become more susceptible to rifampin when infected with certain R factors, those of either the F-like or the I-like type (4). We reported also that such rif-r mutants, when R⁺, are more susceptible to erythromycin, to actinomycin D, and to some rifamycin derivatives which, because they possess a free carboxyl group on the side chain of the rifamycin molecule, do not penetrate efficiently into gram-negative bacteria (3). Since the molecular targets of erythromycin, actinomycin D, and rifamycins are different, and since the ribonucleic acid polymerases extracted from isogenic R⁻ and R⁺ strains are equally susceptible to rifampin, we concluded that R factors increase the permeability of this class of rif-r mutants to these three antibiotics (3, 4).

This observation (that some R factors among our collection of strains increase the sensitivity of these rif-r mutants to some antibiotics) could become of practical interest if the phenomenon also applies to R factors in natural bacterial populations. We decided, therefore, to study the frequency distribution, in natural populations, of R factors capable of increasing the susceptibility to rifampin of this class of rif-r mutants. We isolated 117 gram-negative strains from pathological materials and tested them for presence of R factors transferable to K-12. The 46 strains found to carry R factors were conjugated to strain AJ4, a rif-r mutant belonging to the class of mutants whose susceptibility to rifampin is affected by R factors (3, 4), and the susceptibility of the R⁺ exconjugants was compared with that of the R⁻ AJ4 strain. Since we wanted to work with the same rif-r mutant already used in previous work (3, 4), we were obliged to transfer the R factors in two steps: (i) to a chromosomally resistant auxotroph; and (ii) from the auxotroph to AJ4.

MATERIALS AND METHODS

Strains used. L286 is an F⁻nal⁻ derivative of J53 whose characters are metF⁻ proA⁻. L286(R163 drr) R163 has the characters col I km fi⁻. L362 is an F⁻ str-r derivative of J52 whose characters are proA⁻ his⁻ trp⁻ lac⁻. AJ4 is a merodiploid strain with a nonsense mutation in the rif chromosomal gene and with an F' named KLF10. The chromosomal characters of AJ4 are argG⁻ metB⁻ lacZ⁻, 14 recA-, rif₀; the characters of KLF10 are metB⁺ rif⁻.

The origin of these strains has been reported previously (4). Strains were cloned, and a clone of each was lyophilized after verification of its genotype. Experiments were done with resuspended lyophilized cultures.

Identification of isolates. Identification up to the genus level was carried out by means of Enterotube (Roche).

Susceptibility of isolated strains. The susceptibility of the strains was determined by streaking a loopful of an overnight culture of each of the 117 isolates on agar plates containing Penassay agar (Difco) and one of a number of different antibiotics: tetracycline (10 µg/ml); chloramphenicol (10 µg/ml); kanamycin (5 µg/ml); ampicillin (15 µg/ml); or nalidixic acid (10 µg/ml).

Test for resistance transfer. Eighty-three strains showing resistance to at least one of the tested antibiotics were assayed for transferable resistance by crossing either with L286, in the case of 47 strains that were susceptible to nalidixic acid, or with a str-r mutant derived from L362 in the case of 16 strains resistant to nalidixic acid but susceptible to strep-
tomycin. Exponential cultures of the potential donors, grown in Penassay broth, were crossed with overnight cultures of the appropriate recipient strain. A 0.5-ml amount of donor and 4.5 ml of recipient were incubated 4 h at 37 C; this mating mixture was then plated on selective media: (i) Penassay broth, nalidixic acid (10 µg/ml), and antibiotic to select for a specific transferable factor in crosses with L286; and (ii) Penassay broth, streptomycin (500 µg/ml), and antibiotic to select for a specific transferable factor in crosses with L362.

Test of the effect of R factors on the expression of rifampin susceptibility. The 46 R+ exconjugants obtained from the crosses described above were crossed to AJ4. A 0.05-ml amount of overnight cultures of the 46 donors was added together with 0.45 ml of a culture of the recipient AJ4 to 20 ml of Penassay broth in 200-ml flasks, which were incubated overnight at 37 C. This mating mixture was then plated on two sets of plates, one set containing Davis minimal medium, arginine, and antibiotic (to select for R+ exconjugants), and the other containing, in addition, 200 µg of rifampicin per ml. Colonies were counted after 48 h of incubation at 37 C. As a control, L286(R163) was crossed with AJ4 and similarly tested (4). The ratio of the number of colonies on plates with rifampin to the number in the absence of rifampin is a measure of the increase in susceptibility to rifampicin induced by the introduced R factors.

RESULTS

Table 1 shows the identification, up to the genus level, of 117 recent pathological isolates. The largest group is represented by the genus Escherichia (80 strains). Fifty-five of these strains were resistant to at least one of the following antibiotics: tetracycline, chloramphenicol, kanamycin, ampicillin, and nalidixic acid. Only 35 strains had a resistance transferable to K-12. The second largest group is represented by Aerobacter, with nine transferable resistances among 24 isolates. These results confirm the high frequency of R factors among gram-negative pathogens (1). This is a minimal estimate, since transfer only to K-12 of only one antibiotic resistance was tested.

The strain AJ4 used as recipient carries a rif-r mutation that is highly affected by the presence of R163 (4). Figure 1 shows the plating efficiency (ordinate) of AJ4 and of the exconjugant AJ4 (R163) obtained from a cross of L286 (R163) × AJ4 on agar plates containing increasing concentrations of rifampin (abscissa). The two curves have different shapes and are not parallel. Therefore it is impossible to express with a single figure the ratio of the susceptibility to rifampin of the two strains. The ratio of susceptibility is indeed different at different concentrations of rifampin. To simplify, we selected the concentration of 200 µg of rifampin per ml for a comparison of the relative plating efficiency of R+ recombinants. To test the generality of the ability of R factors to increase the susceptibility to rifampin of a rif-r mutant, the L286 or L362 exconjugants carrying each of the 46 transferable resistances were crossed with the rif-r strains AJ4. The plating efficiencies of the 46 conjugants, AJ4 (R1 through R46), on minimal selective medium (Davis medium, arginine, and an antibiotic selecting the R factor) with

<table>
<thead>
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<th>Genus</th>
<th>No. of isolates</th>
<th>No. of strains resistant to at least one antibiotic</th>
<th>No. of strains with resistance transferable to K-12</th>
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</thead>
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<tr>
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<td>35</td>
</tr>
<tr>
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<td>18</td>
<td>9</td>
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<td>1</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Unidentified</td>
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<td>1</td>
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</table>

![Fig. 1. Plating efficiency of AJ4 on plates containing minimal medium, arginine, and increasing concentrations of rifampin and plating efficiency of AJ4 (R163) recombinants (obtained from a cross of L286 (R163) × AJ4) on plates of minimal medium, arginine, kanamycin (500 µg/ml), and increasing concentrations of rifampin as indicated in abscissa.](http://aac.asm.org/Downloaded from http://aac.asm.org/)
and without 200 μg of rifampin per ml were measured (Fig. 2). All the R factors tested showed an effect on the resistance to rifampin; in fact, the plating efficiency of all the conjun-
gants was less than 1.0. However, it was lower than 0.1 in five strains only. None of the newly isolated, naturally occurring R factors showed an effect as great as that of R163. This effect was independent of the antibiotic used to select the R+ recombinants (which was tetracycline in 19 cases, chloramphenicol in 14, ampicillin in 7, and kanamycin in 6).

DISCUSSION
All the 46 R factors studied, when transferred to the rif-r mutant AJ4, reduced its plating efficiency on rifampin. Thus they behaved like previously studied R factors of our collection of strains (3, 4). We consider the lowered plating efficiency of the rif-r R+ recombinants to be due to decrease in rifampin resistance. Other possible explanations can be ruled out. For example: (i) the reduced plating of exconjugants on selective antibiotics plus rifampin may be due to either curing or to reduced expression of the R resistance, both induced by rifampin (2). This might render a fraction of the conjun-
gants susceptible to the antibiotic that is added to select for the R factor. This explanation, however, can be rejected since clones of AJ4 (R163) grown in the absence of the antibiotic selective for the R factor have a reduced minimal inhibitory concentration for rifampin in broth as compared with clones of AJ4 R- (3, 4). (ii) Another hypothesis made unlikely by the studies of individual clones is that the reduced plating efficiency is due to the synergistic inhibition by the two antibiotics. This seems unlikely since the reduced plating efficiency occurs with various selective antibiotics. (iii) That the reduced plating efficiency of R+ recombinants may be due to some effect operating either on recent exconjugants or is an effect of plating is untenable because established clones of rif-r R+ recom-
binants grown in liquid media also appear to be more susceptible to rifampin than are isogenic R- clones.

As we discussed previously (3, 4), the decrease of resistance to rifampin can be explained by a permeability modification induced by the R factors. However, the effect shown by the majority of wild R factors is modest: only 10% (5 of 46) reduce significantly (to less than one-tenth) the plating efficiency of AJ4 in the presence of 200 μg of rifampin per ml, and in no case is the reduction of such magnitude as to restore the wild-type susceptibility to rifampin. Thus this effect seems unlikely to be of real interest in relation to therapy of patients with rifampin.

The effect of the R factors could, however, have a certain epidemiological importance. If rif-r mutations of the class typified by AJ4 are frequently selected in the course of rifampin therapy of infections due to gram-negative bac-
teria, one might expect rif-r R+ recombinants to be at a disadvantage in comparison with corre-
sponding rif-r R- mutants and therefore less likely to proliferate. This hypothesis would predict that the frequency of R+ strains among rif-r mutants of gram-negative pathogens would be lower than among rif-s strains, and that the majority of the rif-r mutations among rif-r R+ strains would not belong to the class typified by AJ4.

LITERATURE CITED