Third Type of Plasmid Conferring Gentamicin Resistance in
Pseudomonas aeruginosa

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Received for publication 5 May 1975

R1033 is a plasmid of compatibility group P (\(\sim\) P1) transferred from a wild
strain of Pseudomonas aeruginosa. It confers resistance to gentamicin by
gentamicin acetyl-transferase 1 and to kanamycin and neomycin by neomycin
phosphotransferase 1.

The self-transmissible R factors of Pseudomo-
nas aeruginosa clinical isolates have been as-
signed to three compatibility groups by Bryan
and his collaborators (2–4). Group P1 contains
the R factors that in Escherichia coli K-12
constitute group P (13). Group P2 plasmids are
not transmissible to E. coli, and plasmids of
group P3 are those assigned to group C (12) or
com6 (6) in the E. coli plasmid classifications.

Since gentamicin is useful in the treatment of
P. aeruginosa infections, attention has been
concentrated on mechanisms by which resis-
tance to this drug can be attained. Isolates of P.
aeruginosa carrying plasmids conferring genta-
micin resistance have been reported (3, 17, 21).
Bryan et al. (3) found among North American
isolates gentamicin resistance conferred by
plasmids of groups P2 and P3, and Jacoby (17)
has observed such R factors of group P2. In
France, gentamicin R factors of group P3 have
been observed in P. aeruginosa (21).

We report here the isolation, in Spain, of a
strain resistant to gentamicin (and several other
antibiotics) by virtue of an R factor of group P1.

MATERIALS AND METHODS

Bacteria. The bacteria used were P. aeruginosa
CRT and E. coli K-12 strains J53-2 (F\(^{-}\), pro,met,rif),
J62 (F\(^{-}\), pro,his,try,lac), and CR34Thy (F\(^{-}\), thr,leu,
thi,thy,lac).

R factors. The R factors used were Plac, a plasmid
of compatibility group C (\(\sim\) P3) which confers lactose
fermentation ability and sulfonamide resistance (15),
and R751, a plasmid of compatibility group P (\(\sim\) P1)
which confers resistance to trimethoprim (18).

Phage. The phage used was PR1, which adsors
to bacteria carrying plasmids of group P (\(\sim\) P1) and
not to plasmid-free bacteria or cells carrying plasmids
of other groups (11, 19).

Conjugal plasmid transfer and plasmid compat-
ibility properties. Procedures for conjugal plasmid
transfer and the determination of plasmid compat-
ibility properties were as described previously (9, 13).

Minimal inhibitory concentrations. The drugs
used and their minimal inhibitory concentrations are
listed in Table 1.

Radionlabeling and lysis of CR34Thy (R1033) and
isolation of plasmid DNA. Radionlabeling and lysis
of CR34Thy (R1033) and isolation of plasmid deoxyribo-
nucleic acid (DNA) by cesium chloride-ethidium brom-
ide density gradient centrifugation were as de-
scribed previously (7).

Neutral sucrose gradient analysis and calculation
of plasmid molecular weight. Neutral sucrose
gradient analysis and calculation of plasmid mol-
ecular weight were as described previously (1).

Preparation of cell-free extracts. R1033 in E. coli
was grown to late log phase in yeast extract-tryptone
medium (100 ml). The cells were harvested by centri-
figation and washed twice with a solution of 10 mM
tris(hydroxymethyl)aminomethane-hydrochloride (pH
7.8), 50 mM NH\(_4\)Cl, 10 mM MgCl\(_2\), and 0.64 mM
2-mercaptoethanol. The pellet was resuspended in 4
ml of the same buffer and disrupted by sonic treat-
ment. The cell debris was removed by centrifugation
at 100,000 \(\times\) g for 90 min. The resulting supernatant
was designated the sonic extract and is the prepara-
tion that was used in all radioactive assays.

Enzymatic assays. Phosphorylation of aminogly-
coside antibiotics was assayed by the phosphocel-
lulose paper binding assay, as described previously
(20). Acetylation of aminoglycoside antibiotics was
also assayed by the phosphocellulose paper binding
assay, as described previously (5).

RESULTS

Properties of P. aeruginosa CRT. The
strain was pigmented and showed the typical
characters of P. aeruginosa (10). The minimal
inhibitory concentrations of relevant antibiotics
are shown in Table 1.

Strain CRT is unstable, producing segregants
with increased susceptibility to gentamicin
(kanamycin, carbenicillin, tetracycline, strep-
Table 1. Minimal inhibitory concentrations (MIC) of antibiotics for P. aeruginosa CRT and E. coli K-12, with and without R factor R1033

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genta</td>
</tr>
<tr>
<td>P. aeruginosa CRT</td>
<td>500</td>
</tr>
<tr>
<td>P. aeruginosa CRTS*</td>
<td>1.5</td>
</tr>
<tr>
<td>E. coli K-12 strain J53</td>
<td>0.1</td>
</tr>
<tr>
<td>E. coli K-12 strain J53 (R1033)</td>
<td>12.5</td>
</tr>
</tbody>
</table>

* The figures show the lowest concentrations of drug (micrograms per milliliter) in Oxoid DST (direct sensitivity test) agar with added 4% lysed horse blood that prevented visible growth from inocula that gave dense, but not confluent, growth on drug-free control plates. The MIC of butirosin was not measured, but the diameters of the zones of inhibition of E. coli K-12 R⁻ and R1033⁺ round filter paper disks containing 20 μg of butirosin were identical. Abbreviations: Genta, gentamicin; Tobra, tobramycin; Kana, kanamycin; Chlor, chloramphenicol; Carb, carbenicillin.

P. aeruginosa CRTS is the wild strain after spontaneous loss of resistance (see text).

tomycin, chloramphenicol, and sulfonamides). Such segregants have, presumably, lost the R factor. One of these, strain CRTS, is included in Table 1.

When strain CRT was grown with E. coli J53-2, resistance to gentamicin, kanamycin, ampicillin, tetracycline, streptomycin, chloramphenicol, and sulfonamides was transferred. Since all resistances were regularly co-transferred and in view of the compatibility properties and molecular characteristics of the resistant strains, it was concluded that all of the resistances were determined by a single plasmid, R1033.

Properties of R1033 in E. coli K-12. In E. coli, R1033 conferred resistance to gentamicin, kanamycin, ampicillin, tetracycline, and chloramphenicol, and to very low levels of streptomycin and sulfonamides. It confers sensitivity to phage PR1.

J62 (R1033) was mated with CR34Thy(Plac). Transfer in both directions was observed without elimination, and both plasmids were stable in each strain. Thus, R1033 is not a member of group C.

When R751 was transferred into J53-2 (R1033), all the resistance markers of the resident plasmid were eliminated. When R1033 was transferred into J62 (R751), trimethoprim resistance was eliminated (whether selection was made for transfer of ampicillin, gentamicin, or kanamycin). Thus, R1033 is a member of group P (= P1).

Antibiotic-modifying enzymes found in R1033⁺ in E. coli. When sonic extracts of R1033 were assayed for phosphorylating activity, it was found that neomycin and lividomycin were good acceptors for the phosphate moiety but that butirosin was not. This indicates the presence of a phosphotransferase that fits into the neomycin phosphotransferase 1 category (Table 2).

When sonic extracts of R1033 in E. coli were assayed for acetylating activity, it was found that gentamicin C antibiotics and sisomicin were good acceptors for the acetate moiety (Table 3). On the basis of substrate specificities, we have assigned the acetyltransfer found in R1033 in E. coli to the gentamicin acetyltransferase 1 category (5).

R1033 determines production of a TEM β-lactamase and this enzyme is responsible for the resistance to β-lactam antibiotics (M. Matthews and R. W. Hedges, submitted for publication).

Molecular properties of R1033. The molecular weight of R1033, isolated as the covalently closed circular DNA tertiary form by cesium chloride-ethidium bromide density centrifugation, was determined by comparing its sedimentation rate through a neutral sucrose gradient with covalently closed circular R1 plasmid DNA. Figure 1 shows the sedimentation of ³H-labeled R1033 DNA together with ¹⁴C-labeled R1 DNA. Sedimentation was from right to left. The covalently closed circular DNA forms of R1033 and R1 peaked at fractions 19 and 15, respectively, and their own circular forms (formed by spontaneous degradation of the covalently closed circular DNA) peaked at 30 and 28, respectively. By using the value 60 x 10⁴ for the molecular weight of R1 (8; P. T. Barth, unpublished data), the molecular weight of R1033 was calculated to be 45 x 10⁴.

**DISCUSSION**

R1033 in E. coli is found to code for two antibiotic-modifying enzymes. The neomycin phosphotransferase 1 confers resistance to neomycin, kanamycin, and lividomycin, and the gentamicin acetyltransferase 1 confers re-
TABLE 2. Efficiency of different antibiotics as substrates for neomycin phosphotransferase found in R1033 in E. coli$^a$

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>%</th>
</tr>
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<tbody>
<tr>
<td>Neomycin B</td>
<td>100</td>
</tr>
<tr>
<td>Paromamycin</td>
<td>80</td>
</tr>
<tr>
<td>Ribostamycin</td>
<td>63</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>48</td>
</tr>
<tr>
<td>Butirosin A</td>
<td>15</td>
</tr>
<tr>
<td>Butirosin B</td>
<td>13</td>
</tr>
<tr>
<td>Lividomycin A</td>
<td>162</td>
</tr>
<tr>
<td>Lividomycin B</td>
<td>149</td>
</tr>
<tr>
<td>Gentamicin A</td>
<td>53</td>
</tr>
<tr>
<td>Gentamicin B</td>
<td>45</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>3</td>
</tr>
</tbody>
</table>

$^a$ Each compound was incubated with the sonic extract obtained from R1033 in E. coli. Incubation was for 30 min, and results are expressed relative to the phosphorylation of neomycin B (100%).

TABLE 3. Efficiency of different antibiotics as substrates for gentamicin acetyltransferase found in R1033 in E. coli$^a$

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin C$_a$</td>
<td>100</td>
</tr>
<tr>
<td>Gentamicin B</td>
<td>47</td>
</tr>
<tr>
<td>Sisomicin</td>
<td>175</td>
</tr>
<tr>
<td>Neomycin B</td>
<td>0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>30</td>
</tr>
<tr>
<td>Kanamycin A</td>
<td>2</td>
</tr>
<tr>
<td>Kanamycin B</td>
<td>53</td>
</tr>
<tr>
<td>Kanamycin C</td>
<td>5</td>
</tr>
<tr>
<td>Butirosin A</td>
<td>0</td>
</tr>
<tr>
<td>Lividomycin A</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>8</td>
</tr>
</tbody>
</table>

$^a$ Each compound was incubated with the sonic extract obtained from R1033 in E. coli. Incubation was for 30 min. Results are expressed relative to the acetylation of gentamicin C$_a$ (100%).

Resistance to gentamicin C$_a$ compounds, sisomicin, and kanamycin B. An interesting result is that the acetylation enzyme found in R1033 in E. coli will utilize tobramycin as a substrate, but that the cells are found to be susceptible to tobramycin in vitro. This is not surprising in view of the fact that many acetylated aminoglycoside antibiotics are still potent antibiotics (M. J. Haas, personal communication).

R1033, of molecular weight $45 \times 10^6$, is larger than any P plasmid previously reported. RP4 has a molecular weight of $34 \times 10^6$ (14), that of R906 is $35 \times 10^6$ (16), that of R751 is $30 \times 10^6$ (P. T. Barth and N. J. Grinter, personal communication), and that of RW4a is $38 \times 10^6$ (A. E. J. and R. W. H., unpublished data). R1033 is the first plasmid of group P1 shown to confer resistance to gentamicin or chloramphenicol. Perhaps it carries a DNA sequence derived from some unknown source, which confers these resistances and augments the molecular weight of the plasmid.

ACKNOWLEDGMENTS

A. E. Jacob was supported by a grant to Naomi Datta from the Medical Research Council.

LITERATURE CITED


