Plasmid-determined Fusidic Acid Resistance in the Enterobacteriaceae

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SUMMARY

A number of R factors have been shown to determine resistance to fusidic acid, an antibiotic effective against Gram-positive bacteria. Among several R factors examined for this property only one was fi^- ; the remainder were fi^+ and of the FII compatibility group.

INTRODUCTION

Fusidic acid is a steroidal antibiotic which has been used in the treatment of staphylococcal infections, especially when penicillin resistance is a problem (Garrod, Lambert & O'Grady, 1973). Reports of fusidic acid resistance among clinical isolates of Gram-positive organisms are rare. In two cases resistance seemed to be determined by plasmids in *Staphylococcus aureus* (Evans & Waterworth, 1966).

Fusidic acid has no therapeutic application for Gram-negative bacteria because they are insensitive to the drug at concentrations attainable in body fluids. However, fusidic acid is a useful inhibitor of protein synthesis in bacteria and it has been studied extensively in appropriate strains of *Escherichia coli*. Mutants resistant to high concentrations, have been isolated and shown to have an altered translocation factor (EF_G); the drug interferes with the binding of aminoacyl-tRNA to ribosomes and is useful in studies of certain complexes involved in protein synthesis (Pestka, 1971; Cundliffe, 1972; Davies & Nomura, 1972). *E. coli* mutants more than usually sensitive to fusidic acid have been isolated, and we presume that these mutants have altered cell envelopes, since they are also permeable to other agents which do not normally affect *E. coli* (Ennis, 1971; D. Becker and J. Davies, unpublished).

Since fusidic acid resistance is believed to be plasmid-determined in *S. aureus*, we tested whether any R factors of Enterobacteriaceae carried a fusidic acid-resistance determinant by transferring a variety of R factors into fusidic acid-sensitive mutants of *E. coli* and screening for resistance to the drug.

METHODS

Preparation of fusidic acid-sensitive strains of E. coli. *E. coli* PR7 F^- *thi leu rns pnp nal-r* (Taylor, 1970) was grown to exponential phase in yeast extract-tryptone medium (ML) and treated with 100 μ g *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/ml at 37 °C for 5 min. The cells were collected by centrifuging, washed with ML medium, resuspended in the same medium and streaked on ML-agar plates. Colonies were picked on to ML plates with sterile toothpicks

and replica-plated to a series of ML plates containing ($\mu\text{g/ml}$): fusidic acid, 100; streptolydigin, 100; novobiocin, 100; clindamycin, 100. Strains sensitive to these antibiotics were isolated, purified and checked for auxotrophic markers. Most of the fusidic acid-sensitive mutants were also sensitive to the other drugs tested. *E. coli* DB10 is one such strain; a disc of fusidic acid (20 μg) gave a zone of inhibition of 18 mm on ML plates.

Transfer of fusidic acid resistance. R factor-containing strains and the recipient *E. coli* DB10 were grown to mid-exponential phase in ML broth and equal volumes were mixed and allowed to stand at 37 °C for 1 h. The mixed culture was streaked on ML plates containing nalidixic acid (30 $\mu\text{g/ml}$) and one of the antibiotics [usually neomycin (20 $\mu\text{g/ml}$) or kanamycin (30 $\mu\text{g/ml}$)] to which the R factor conferred resistance. After incubation for 24 h, colonies were picked and checked for nutritional and resistance markers. Resistance patterns were scored by disc tests; a disc of fusidic acid (20 μg) gave a zone of inhibition of 17 to 19 mm on a sensitive strain, 8 to 10 mm on a resistance strain. No R factor-determined resistance to streptolydigin, novobiocin or clindamycin was detected. Fusidic acid resistance in mutant or wild-type *E. coli* K12 was also demonstrated using agar ditches containing various concentrations of fusidic acid.

Compatibility properties. These were determined by the techniques described by Datta *et al.* (1971) and Coetzee, Datta & Hedges (1972).

Bacterial strains. F⁻ *E. coli* K12 strains, PR7 and its variant DB10 (described above), J53 *pro met*, J53.2 (a rifampicin-resistant mutant of J53), and J62 *pro his trp lac*, were used.

Plasmids. These are listed in Table 1. The isolation of plasmids lacking one or more resistance markers was as described by Coetzee *et al.* 1972.

RESULTS

Of ten different R factors routinely screened at the start of the work, JR70, JR72 and JR73 conferred fusidic acid resistance. All three were fi⁺. All eliminated R1-I efficiently when transferred into a strain carrying that plasmid.

JR73 was efficiently eliminated when R1-I was transferred into a JR73⁺ strain (20 of 20 transconjugants had lost resistance to kanamycin and tetracycline) and was thus a typical FII plasmid; JR70 was also eliminated by transfer of R1-I but the elimination was less efficient, only two of 20 transconjugants had lost JR70 resistances in the usual test screening but the doubles were unstable. When the doubles were grown in antibiotic-free medium, segregants lacking either one of the plasmids were observed, though R1-I⁻ segregants were more abundant than JR70⁻.

This pattern resembles the relationship observed by MacFarren & Clowes (1967) between the F factor and ColV⁺ factors. They found that transfer of an F factor into a ColV⁺ strain efficiently eliminated the resident plasmid but that the reciprocal transfer led to elimination of the F factor only after long delay.

JR72 behaved differently. When R1-I was transferred into a strain carrying JR72, no transconjugants lacking the resident plasmid were observed (Table 2). The transconjugants were doubles which produced R1-I⁻ segregants in antibiotic-free media but JR72⁻ segregants were never observed. When such an unstable double was transferred to nutrient broth containing 1000 μg ampicillin/ml (an antibiotic to which R1-I, but not JR72, determines resistance) and incubated overnight, all the viable progeny tested (242 of 242) retained kanamycin resistance (a marker of JR72).

Table 1. *Plasmids used in this work*

Abbreviations: A, ampicillin; S, streptomycin; T, tetracycline; C, chloramphenicol; K, kanamycin; Su, sulphonamide; Tp, trimethoprim; Gk, gentamicin/kanamycin.

Designation	Properties	Resistances	Compatibility	Source or reference
JR70*	+	S, T, C, K, Su	FII	<i>E. coli</i> JR35
JR72*	+	S, C, K, Su	FII	<i>E. coli</i> NIHJ268
JR73*	+	S, T, C, K, Su	FII	
R1-1*	+	A, S, C, Su	FII	Hedges & Datta (1972)
R28*	+	S, T, Su	FII	Lawn, Meynell, Meynell & Datta (1967)
R136	+	T	FII	Lawn <i>et al.</i> (1967)
222*	+	S, T, C, Su	FII	Watanabe <i>et al.</i> (1964)
R494	+	A, T, K	FII	Hedges, unpublished
<i>Flac</i>	(-)		FI	Dennison (1972)
R386	+	T	FI	Dennison (1972)
R455	+	A, S, T, C, Su	FI	Hedges, Datta, Coetzee & Dennison (1973)
ColB-K98	+		FIII	Frydman & Meynell (1969)
R124	+	T	FIV	Hedges & Datta (1972)
R64-1	-	A, S, T,	Ix	Datta <i>et al.</i> (1971)
R46	-	A, S, T, Su	N	Datta & Hedges (1971)
R388	-	Su, Tp	W	Datta & Hedges (1972a)
R483	-	S, Tp	I ϕ	Hedges & Datta (1973)
R57b*	-	A, C, Gk, Su	C	Datta & Hedges (1972b)
RP4	-	A, T, K,	P	Datta <i>et al.</i> (1971)
R401	-	A, S	T	Coetzee <i>et al.</i> (1972)
R6K	-	A, S	X	Hedges <i>et al.</i> (1973)
RA1	-	T, Su	A	Datta & Hedges (1973)

* These plasmids confer fusidic acid resistance.

Table 2. *Compatibility properties of JR70 and JR72*

Abbreviations: K, kanamycin; A, ampicillin; T, tetracycline; pro, proline; his, histidine; trp, tryptophan; met, methionine; ^s, sensitive; ^R, resistant.

Donor	Recipient	Selective medium	Transfer rate	Properties of transconjugants
J53.2 (JR72)	J62	pro, his, trp, K	7×10^{-2}	—
J53.2 (JR72)	J62 (R1-1)	pro, his, trp, K	4×10^{-2}	18A ^s K ^R :2A ^R K ^R *
J62 (R1-1)	J53	pro, met, A	8×10^{-3}	—
J62 (R1-1)	J53 (JR72)	pro, met, A	3×10^{-3}	40/40 A ^R K ^R
J53.2 (JR70.1)	J62	pro, his, trp, T	1×10^{-2}	—
J53.2 (JR70.1)	J62 (JR72)	pro, his, trp, T	6×10^{-3}	40/40 T ^R K ^R
J62 (JR72)	J53.2	pro, met, K	1×10^{-1}	—
J62 (JR72)	J53.2 (JR70.1)	pro, met, K	4×10^{-2}	20/20 T ^s K ^R

* These were stable and found to carry recombinant R factors with the compatibility properties of JR72 and (at least) the ampicillin resistance gene of R1-1.

One of these clones was tested and found to be carrying a recombinant plasmid with the resistance markers of both R1-1 and JR72.

The ability of JR72 and R1-1 to form recombinants indicates extensive DNA homology between them. Several such recombinants have been observed. One [detected as a transconjugant that retained ampicillin resistance after JR72 was transferred to J62(R1-1)] was studied in greater detail.

This strain was mated with J53(R136). When selection was made for transfer of ampicillin or kanamycin resistance into J53(R136), in all cases (40 of 40) the transconjugants were resistant to ampicillin and kanamycin but sensitive to tetracycline (i.e. R136 was eliminated). When R136 was transferred into the strain carrying the recombinant plasmid, trans-

Table 3. *Incompatibility of segregants of JR72*

JR72-1 is a segregant of JR72 which lacks the chloramphenicol-resistance determinant. JR72-2 is a segregant of JR72 which lacks the kanamycin-resistance determinant. C, Chloroamphenicol; for other abbreviations see Table 2.

Donor	Recipient	Selective medium	Character of transconjugants
J53.2 (JR72-1)	J62 (JR72-2)	pro, his, trp, K	20/20 C ^S K ^R
J62 (JR72-1)	J53.2 (JR72-2)	pro, met, K	20/20 C ^S K ^R
J53.2 (JR72-2)	J62 (JR72-1)	pro, his, trp, C	20/20 C ^R K ^S
J62 (JR72-2)	J53.2 (JR72-1)	pro, met, C	19C ^R K ^S : 1C ^R K ^{R*}

* This strain was an unstable double, segregating both C^S and K^S progeny.

conjugants all (20 of 20) retained ampicillin and kanamycin resistance. Thus, the recombinant plasmid has the compatibility properties of JR72 (not R1-1, which would have been eliminated by R136). This was predictable since the newly formed recombinant plasmid had to be able to compete with JR72 in order to survive.

Transfer of JR72 into cultures carrying representative plasmids of groups FI (R455), FIII (colB-K98) or FIV (R124) did not eliminate the resident plasmid. Thus, JR72 is not a member of any of these groups.

Compatibility of JR72 and JR70

Since JR70 seemed intermediate in susceptibility to elimination between JR72 and R1-1 (being eliminable by R1-1, but not readily so), we investigated the relationship between JR70 and JR72 by isolating a segregant of the former, JR70-1, which had lost all resistance markers (including fusidic acid resistance) except tetracycline resistance, but was still self-transmissible. In crosses with JR72, JR70-1 behaved precisely as did R1-1 (Table 2), i.e. transfer of JR72 eliminated R1-1 efficiently, whereas transfer of JR72 into a JR70-1⁺ strain led to the production of doubles which were stable for JR72 but readily segregated JR70-1⁻ clones in the absence of selection for retention of the latter plasmid.

Elimination of segregants of JR72

A possible explanation of the behaviour of JR72 would be some special barrier to its elimination. To test this possibility, segregants of JR72, one lacking chloramphenicol resistance and the other lacking kanamycin resistance, were isolated. Both segregants retained all testable characters of JR72 other than the single marker. Crosses between strains carrying these segregants (Table 3) revealed that elimination of the segregants proceeded with typical efficiency. Thus there was no barrier to the elimination of JR72.

The possibility that JR72⁻ segregants would be inviable in a JR72⁺ culture (perhaps because the R factor determined production of, and immunity to, a colicin) was eliminated by reconstruction experiments which showed that growth of an R⁻ strain was not inhibited by the presence of an excess of JR72⁺ cells (nor was production of a colicin by JR72⁺ cultures demonstrable).

Plasmids conferring no fusidic acid resistance

Not all F-like plasmids confer fusidic acid resistance, e.g. the segregant JR70-1. Among other FII plasmids tested, R1, R28 and 222 confer resistance to fusidic acid whereas R136 and R494 do not.

F-like plasmids belonging to compatibility groups other than FII (*Flac*, R386 and R455

of group FI, ColB-K98 of group FIII, and R124 of group FIV) did not affect fusidic acid resistance.

Non-F-like plasmids representing nine compatibility groups were tested for ability to confer fusidic acid resistance; R57b was the only one to show such resistance.

Fusidic acid-resistance R factors in normal strains of Escherichia coli K12

By streaking R⁺ and R⁻ *E. coli* strains against ditches containing very high concentrations of fusidic acid it was shown that R factors which conferred fusidic acid resistance upon the sensitive mutant DB10 also increased the resistance of wild-type strains.

DISCUSSION

In a variety of Gram-negative and Gram-positive bacteria, mutations to fusidic acid resistance result in an altered protein synthesis component (EF_G) which is normally the binding site for the drug in exerting its inhibitory action on protein synthesis (Davies & Nomura, 1972). We do not know the mechanism of R factor-coded fusidic acid resistance. It is unlikely to be an alteration of EF_G since the resistance level is low. It could be due to altered permeability or enzymic inactivation of the drug.

That certain R factors should carry a resistance determinant of no apparent use to their normal hosts is of interest, since wild-type Enterobacteriaceae are insensitive to fusidic acid. The resistance is not merely a suppression of the mutant defect, but is expressed in the normal bacterium. One might expect that an apparently useless character would have segregated out during evolution. There are two possible explanations for the presence of this seemingly redundant r-determinant.

(i) Fusidic acid resistance is a gratuitous property associated with another resistance determinant. Resistance to another agent (as yet unknown) may arise by a change which also affects sensitivity to fusidic acid. Perhaps organisms exist which produce steroidal antibiotics whose actions resemble fusidic acid (so that a bacterium may increase its resistance to all such molecules by a single mechanism) and which are active against Gram-negative bacteria at concentrations attainable in nature. The fusidic acid resistance may have evolved as a response to these antibiotics.

(ii) R factors are very stable and can keep their r-determinants even in the absence of selection (e.g. many R factors still carry streptomycin resistance, although streptomycin is not much used in medicine). We therefore suggest that R factors and staphylococcal plasmids are genetically related (they possess several common resistance determinants, in addition to fusidic acid). Perhaps an exchange mechanism exists whereby plasmids, or parts of plasmids, may be exchanged between Gram-positive and Gram-negative strains. Both *E. coli* and *S. aureus* are susceptible to transfection by plasmid DNAs (Cohen, Chang & Hsu, 1972; Lindberg, Sjöström & Johansson, 1972) so that genetic exchange need not involve conjugation between cells but merely the uptake of free DNA molecules by the recipient.

Whatever their origins, the fusidic acid-resistance factors are transmissible R factors in *E. coli*. R factor R57b, a prototype of compatibility group C (Datta & Hedges, 1972*b*) was the only fii⁻ plasmid shown to determine fusidic acid resistance. The others were all fii⁺ and related to compatibility group FII. Some, e.g. R1, are typical members of the group, eliminating and being eliminated by other plasmids of the group, but others are unusually resistant to elimination by members of the group. However, they show other characteristics of group FII, efficiently eliminating or undergoing recombination with resident FII plasmids. Their

compatibility properties resemble those of the mutant R1 plasmids described by Nordström, Ingram & Lundbäck (1972).

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