Increased Resistance to Several Antibiotics by One Mutation in an R-factor, R 1a

By K. NORDSTRÖM

Department of Microbiology, University of Umeå, S-901 87, Umeå 6, Sweden

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SUMMARY

An Hfr strain G 11 and an F⁻ strain D 1 of *Escherichia coli* K 12 both containing the R-factor R 1a were treated with ethyl methane sulphonate (EMS). Mutation of the bacteria to high ampicillin resistance occurred at a frequency of 10^{-3} to 10^{-4} and it was coupled with a simultaneous increase in resistance to chloramphenicol, streptomycin and sulphanilamide. Resistance to all the antibiotics was infective. Increased ampicillin resistance was due to increased penicillinase activity of the bacteria. In a strain containing a mutant R-factor (R 1 B 1) this was due to an increased quantity of an enzyme that seemed to be identical to that of a strain containing the unmutated R 1a. Metabolism of chloramphenicol in strains containing R 1 B 1 was greater than in those with R 1a. Mating experiments with an Hfr strain carrying R 1a or R 1 B 1 revealed that the two R-factors reduced fertility to about the same extent. Pair formation was two to three times greater in the presence of R 1 B 1 than in the presence of R 1a, and R-factor transfer was increased more than chromosome transfer. It is likely that the R 1a genome is read two to three times more efficiently in strains containing the mutated R 1 B 1, perhaps due to an increased number of copies of the R-factor genome per bacterium.

INTRODUCTION

Resistance to antibiotics may be determined by genes located on episomes (R-factors). The amount of DNA in these episomes is small (about 1 % of that in the bacterial chromosome), but they have genes which determine resistance to as many as seven antibiotics. R-factors are transferred from donor to recipient bacteria in a process analogous to conjugation. The transfer is not restricted to single species but can take place between different Enterobacteria (see reviews by Watanabe, 1963, 1967; Datta, 1965; Meynell, Meynell & Datta, 1967; Anderson, 1968; Campbell, 1969). In some cases R-factor mediated resistance has been shown to depend upon metabolism of the drug (Datta, 1965; Okamoto & Suzuki, 1965). Some of the enzymes such as penicillinases are well characterized; others have been only partly purified.

Ampicillin-resistant mutants of *Escherichia coli* K 12 fall into two classes. Those in class I result from mutation of the *ampA* gene and possess a tenfold increase both in resistance (Eriksson-Grennberg, Boman, Jansson & Thorén, 1965) and penicillinase activity (Burman, Nordström & Boman, 1968; Lindström, Boman & Steele, 1970). *AmpA* is located close to *purA* at 82 min. on the K 12 chromosome (Eriksson-Grennberg, 1968). Mutants belonging to class II show a twofold increase in ampicillin resistance on agar medium but do not form an increased amount of penicillinase (Boman, Eriksson-Grennberg, Földes & Lindström, 1967). When R-factors were introduced into *ampA* bacteria the ampicillin resistance effect was additive. If an *ampA* mutation and R-factors were introduced into mutants of class II yet a further increase of resistance was detected. R-factors could therefore be used to identify

class II mutants, e.g. in analysing the results of crosses (Nordström, Eriksson-Grennberg & Boman, 1968). The mutation experiments described in this paper show that two sorts of ampicillin-resistant mutants could be obtained from bacteria containing the R-factor R1a, one which was of the chromosomal class II type and the other which had resulted from mutation in the R-factor. One such mutated R-factor is described in detail. A preliminary report of this work has appeared (Nordström, 1969).

METHODS

Strains, media and materials. All strains used were derived from *Escherichia coli* K12 and are listed in Table 1. The R-factor R1 was obtained from Dr Datta (Meynell & Datta, 1966) and the derepressed mutant R1 *drd-19* (Meynell & Datta, 1967) from Dr Meynell; R1 normally confers resistance to ampicillin, chloramphenicol, kanamycin, streptomycin and sulphonamides. Resistance to kanamycin is easily lost (N. Datta, personal communication) and our R1 was only resistant to the other four antibiotics. It was given the symbol R1a.

Table 1. *Strains of Escherichia coli* K12 used

Strain	Origin	Sex	Ampicillin resistance			Other markers†
			Class	Phenotype*	Genotype	
G II	Stent & Brenner (1961)	Hfr	wt	Amp-s	wt	<i>metB</i> , <i>ilv</i>
G II a1	Eriksson-Grennberg <i>et al.</i> (1965)	Hfr	I	Amp-10	<i>ampA</i>	<i>metB</i> , <i>ilv</i>
G II e1	Eriksson-Grennberg <i>et al.</i> (1965)	Hfr	II	Amp-20	<i>ampA</i> + an additional mutation‡	<i>metB</i> , <i>ilv</i>
D I	RC 711 of Meynell & Datta (1966)	F ⁻	wt	Amp-s	wt	<i>his</i> , <i>proB</i> , <i>trp</i>
D II	Boman, Eriksson-Grennberg, Normark & Matsson (1968)	F ⁻	wt	Amp-s	wt	<i>his</i> , <i>proB</i> , <i>trp</i> , <i>str</i>

* Defined by Nordström *et al.* (1968); the figure indicates the factor by which the wild-type resistance (Amp-s) is increased.

† Abbreviations: *amp*, ampicillin; *his*, histidine; *met*, methionine; *ilv*, isoleucine-valine; *pro*, proline; *str*, streptomycin; *trp*, tryptophan; wt, wild-type.

‡ See Nordström *et al.* (1969).

The minimal medium E of Vogel & Bonner (1956) and the complete medium LB of Bertani (1951) were used, the latter containing 0.2% glucose (w/v). Solid medium was obtained by the addition of 1.5% agar; that derived from LB medium was called LA. All experiments were performed at 37°, and growth was determined by reading extinction with a Klett colorimeter using filter W66. A reading of 100 Klett units corresponds to 4×10^8 bacteria/ml. when grown in LB medium.

DL-Ampicillin (ratio between the epimers 4:6) and D-ampicillin were obtained from Astra, Södertälje, Sweden; chloramphenicol as the free base and streptomycin sulphate from Kabi, Stockholm, Sweden; ethylmethane sulphonate (EMS) from Eastman Organic Chemicals (Eastman grade, catalogue no. 7830); and kanamycin from AB Ferrosan, Malmö, Sweden. The microculture containers used for replica plating were obtained from Elessa, Milan, Italy.

Induction of mutations. The bacteria were grown in LB at 37° on a rotary shaker to a density of about 4×10^8 organisms/ml., EMS was added to a final concentration of 10 µl./ml. and incubation continued for 2 h. The cultures were centrifuged, washed twice with

0.9% (w/v) NaCl and suspended in fresh LB. Incubation was continued for further 2 h. to allow phenotypic expression.

Transfer of R-factors and chromosomal markers. The procedure for the transfer of the R-factor R1 was as described by Nordström *et al.* (1968). In mating experiments 2×10^7 donor and 2×10^8 recipient bacteria/ml. grown in LB and harvested in the log phase were mixed, incubation continued and mating interrupted by blending on a Vortex mixer. Different dilutions of the mating mixture in 0.9% NaCl (w/v) were spread on selective medium and those giving 40 to 400 colonies/plate were used to calculate the frequencies shown in Fig. 4 and 5.

Pair formation was measured in a mating procedure as described by de Haan & Gross (1962); 2×10^7 donor and 2×10^8 recipient bacteria/ml. were mixed and 5 min. allowed for pairing. The culture was then diluted 100-fold with prewarmed medium to prevent further pair formation. Samples were taken at intervals, shaken to separate pairs and spread on selective medium. The plateau value obtained is a measure of the number of pairs formed initially.

Transduction with phage P1 was as described by Eriksson-Grennberg (1968).

Drug resistance. Preliminary tests were carried out by replica plating onto LA medium containing the drug. Due to the high penicillinase activity associated with the R-factors, it was necessary to dilute the inocula to less than 100 bacteria per spot. In single organism tests the bacteria were grown in LB to a density of 4×10^8 /ml., diluted 10^{-5} in 0.9% (w/v) NaCl, and 0.1 ml. plated on LA plates containing different concentrations of antibiotics. The colonies formed were counted after incubation overnight and the resistance level is given as the highest concentration of antibiotics at which the colony count was the same as that of the control (Nordström *et al.* 1968). Tests for resistance to sulphanilamide were done on solid minimal medium.

Penicillinase was determined by means of Novick's (1962) micro-iodometric method adapted to the AutoAnalyzer (Burman *et al.* 1968).

The same paper disc method and the strain of *Sarcina lutea* was used as described previously for the determination of the destruction of penicillin (Burman *et al.* 1968). Chloramphenicol in the concentration range 2 to 30 µg./ml. could also be determined with this method, i.e. the sensitivity was the same for chloramphenicol as for ampicillin.

The procedure used to purify the R1 penicillinase was as described by Lindqvist & Nordström (1970).

RESULTS

Isolation of strains with higher resistance

The R-factor R1a was introduced into G11 and D1, giving strains G11-R1a and D1-R1a respectively. Log-phase cultures of the two latter strains containing 4×10^8 bacteria/ml. were treated with 10 µl. EMS/ml. and then spread on LA plates containing DL-ampicillin at different concentrations as described in the legend to Fig. 1. Untreated cultures were used as controls and the survival curves obtained are shown in Fig. 1. For both strains, EMS treatment yielded a higher proportion of clones with high resistance; 10^{-3} to 10^{-4} of the surviving bacteria as compared with 10^{-5} to 10^{-6} from the untreated cultures. Clones were picked from plates containing 500 µg./ml. of DL-ampicillin and purified by restreaking on plates containing the same concentration of ampicillin.

The clones isolated as resistant to 500 µg./ml. of DL-ampicillin were tested for resistance to ampicillin and chloramphenicol at different concentrations by replica plating. The results together with the resistance levels of the parental strains are given in Table 2. The Hfr strain G11-R1a and the F⁻ strain D1-R1a behaved similarly and two classes of mutants

both showing parallel increased resistance to chloramphenicol were obtained. The clones obtained after EMS treatment showed a considerable increase in resistance, while without EMS treatment clones of lower resistance resembling class II strains were isolated (cf. strain G11e1-R1a in Table 2).

When clones were selected at higher concentrations of DL-ampicillin several classes of mutants were obtained. The higher the concentration of the antibiotic, the higher the resistance of the surviving clones. A small number of clones with different levels of resistance were tested for penicillinase production. Since it was shown (see below) that they were mutated in the R-factor they were denoted D1-R1B1, etc. Table 3 shows that they possessed different penicillinase activities and that the activity was correlated with resistance.

When subcultured, bacteria from the most resistant clones were found to be rather unstable and they reverted to R1a resistance or to an intermediate level. However, some of

Fig. 1. Mutation of strains G11-R1a and D1-R1a to higher ampicillin resistance. Experimental conditions were as described in Methods. Different dilutions were then spread on LA containing different concentrations of DL-ampicillin (abscissa). The number of colonies were counted after incubation overnight and those dilutions that gave 20 to 200 colonies per plate were used to calculate survival.

Table 2. *Resistance to ampicillin and chloramphenicol of mutants picked as resistant to 500 μg./ml. of DL-ampicillin*

Bacteria were grown in LB at 37° on a rotary shaker to a density of about 4×10^8 orgs./ml., EMS added to a final concentration of 10 μl./ml. and incubation continued for 2 h. The cultures were centrifuged, washed twice with 0.9% (w/v) NaCl and suspended in fresh LB. Incubation was continued for a further 2 h. to allow phenotypic expression. Different dilutions were spread on LA containing 500 μg. of DL-ampicillin/ml. From those plates that gave 20 to 200 colonies a number of clones were picked, purified by restreaking on LA and tested for resistance by replication.

Strain	Treatment		No. of clones tested	Resistance (μg./ml.)	
	EMS	Ampicillin plates		DL-Ampicillin	Chloramphenicol
D1-R1a (F ⁻)	-	-	1 (parent)	200	200
	-	+	32	400	400
	+	+	37	≥ 700	≥ 600
G11-R1a (Hfr)	-	-	1 (parent)	200	200
	-	+	34	400	400
	+	+	35	≥ 700	≥ 600
G11e1-R1a (Hfr)	-	-	1 (parent)	400	400

the mutant R-factors were found to be stable and one of them, R1 B1, was studied further. It was transferred from D1 to G11 strains and tested for resistance to all four antibiotics by the single organism test. The results in Table 4 show that resistance to all four antibiotics was increased severalfold. This led to the conclusion that the mutation resulting in increased resistance was located in the episome. In this table the resistance pattern given by Datta's derepressed R-factor R1 *drd-19* is also included since the R-factor R1 B1 could have been a regulatory mutant of R1a.

Table 3. *Penicillin resistance and penicillinase activity of strain D1 containing EMS-induced mutations in the episome R1a*

Six of the mutant clones obtained from D1-R1a in the experiment described in Table 2 were tested for ampicillin resistance by single organism test on LA plates and for penicillinase activity in the AutoAnalyzer.

Strain	DL-Ampicillin resistance ($\mu\text{g./ml.}$)	Penicillinase activity (units/ 10^8 bacteria)
D1-R1a	200	2.9
D1-R1 B1	600	5.8
D1-R1 B2	500	4.8
D1-R1 B3	700	6.9
D1-R1 B4	≥ 1000	12.5
D1-R1 B5		14.8
D1-R1 B6		22.0

Table 4. *Resistance pattern of R-factor strains*

Resistance was tested by single organism tests on LA plates.

Strain	Resistance on plates ($\mu\text{g./ml.}$)			
	D-Amp	Cml	Str	Sul
G11-R1a	75	300	15	300
G11-R1 B1	200	800	40	1000
G11-R1 <i>drd-19</i>	75	300	15	—

Abbreviations used: Amp, ampicillin; Cml, chloramphenicol; Str, streptomycin; Sul, sulphanilamide.

To demonstrate that this mutation could be transferred by infection the clones described in Table 2 were tested for transfer of resistance to an F⁻ recipient. Mutants of both the Hfr strain G11-R1a and the F⁻ D1-R1a were tested as donors. All strains isolated after EMS treatment transferred resistance to high concentrations of antibiotic, while the untreated strains conferred only the lower level of resistance of the parental strain R1a. This confirmed that the EMS-induced mutations were located in the R-factor. Without EMS treatment the mutants isolated were of class II, i.e. similar to strain G11e1-R1a (Table 2) which is mutated in the chromosome (Nordström *et al.* 1968; Nordström, Burman & Eriksson-Grennberg, 1969).

Comparison of resistance conferred by R1a and by R1 B1

Resistance to ampicillin and chloramphenicol was also tested in liquid medium. Strains G11-R1a and G11-R1 B1 were grown in LB medium to a density of about 10^8 organisms/ml. DL-Ampicillin or chloramphenicol was added, incubation continued and the extinction of the cultures recorded. The results are plotted in Fig. 2 and 3 and they show that the mutated R-factor mediated a considerably increased resistance to the two drugs tested as compared

to R1a; G11 without any R-factor does not grow even in the presence of 10 $\mu\text{g}/\text{ml}$. of ampicillin or chloramphenicol. Also at concentrations of ampicillin or chloramphenicol that inhibited the growth of both strains, growth was resumed much more rapidly by G11-R1B1 than by G11-R1a. Such curves are normally interpreted as an indication that the drug tested is being broken down. In a bioassay using *Sarcina lutea* it was shown that the strain G11-R1B1 broke down chloramphenicol about twice as rapidly as did strain G11-R1a. The penicillinase activity of strain G11-R1B1 was much greater than that of G11-R1a (Table 3).

Fig. 2

Fig. 2. Effect of ampicillin on growing cultures of G11-R1a and G11-R1B1. The strains were grown in LB medium. Ampicillin was added when the cultures contained 10^8 organisms/ml., incubation was continued and the extinction was read at intervals over a period of 2 h. The concentrations of ampicillin used are given in $\mu\text{g}/\text{ml}$. in the figure.

Fig. 3

Fig. 3. Effect of chloramphenicol on growing cultures of G11-R1a and G11-R1B1. Experimental conditions were as described in Fig. 2.

Purification of penicillinase

The penicillinase produced by the R-factor R1a has been purified (Lindqvist & Nordström, 1970) and it is very similar to the enzyme produced by R1B1. The most important finding is that the turnover numbers are the same for the two enzymes and that the amount of the enzyme per bacterium was doubled by the mutation.

Sex properties of bacteria carrying R1a and R1B1

The mating characteristics of strains carrying the R1a and the mutated R1B1 factors were compared. Fig. 4 shows the effect of each of the two R-factors on the transfer of an early gene (*proB*⁺) from Hfr G11 to the F⁻ strain D11. Both R-factors greatly reduced the transfer of chromosomal genes and it was necessary to multiply the numbers of recombinants obtained in crosses in which the donor contained an R-factor by 10 in Fig. 4. Nevertheless, *proB*⁺ transfer by strain G11-R1B1 was about twice that for G11-R1a. Recombinants obtained in the two crosses were also tested for cotransfer of episomal markers with chromosomal genes. In both cases, 46 *proB*⁺ recombinant clones obtained after a mating time of 40 min. were purified and tested by replica plating. The first cross (D11 × G11-R1B1) gave no cotransfer of resistance with *proB*⁺ while two cases appeared in the latter (D11 ×

G11-R1a). There was a slightly delayed entry in the crosses in which donor contained R-factors (Fig. 4). The significance of this is not understood.

In the same experiment as that reported in Fig. 4 the kinetics of the transfer of antibiotic resistance from G11-R1a and G11-R1B1 to the recipient D11 was also studied. As is apparent from Fig. 5, the transfer of the mutated R-factor R1B1 was about 10 times as efficient as that of R1a. This means that pair formation was more efficient with G11-R1B1 than with G11-R1a.

Using a more direct measure of pair formation (de Haan & Gross, 1962) R1B1 gave two to three times more pairs than R1a (Table 5). Furthermore, R-factor transfer was much greater once pairs had been formed.

Fig. 4

Fig. 5

Fig. 4. Kinetics of the formation of *proB*⁺ recombinants in crosses between the Hfr strain G11 without and with R-factors and the F⁻ strain D11. Donor (2×10^7 /ml.) and recipient bacteria (2×10^8 /ml.) were mixed and incubated without agitation at 37°. At intervals samples were shaken vigorously to break all pairs and plated on selective medium with streptomycin (100 µg./ml.) to eliminate the donor. The number of recombinants from crosses in which the donor contained an R-factor have been multiplied by 10.

Fig. 5. Kinetics of transfer of the R-factors R1a and R1B1. The experiment was as described in Fig. 4. Selection was made on minimal medium plus the amino acids required by the recipient. Chloramphenicol (100 µg./ml.) was used to eliminate the recipient cells which had not received the R-factor.

Table 5. Pair formation with strains containing R-factors

Matings were carried out by the method of de Haan & Gross (1962). The table gives the frequencies of recombinants when plateau values were reached.

Cross	Plateau values (% of donor) for transfer of		
	<i>proB</i> ⁺	R-factor	Sum
D11 × G11	2.10	—	2.10
D11 × G11-R1a	0.06	0.02	0.08
D11 × G11-R1B1	0.10	0.18	0.28

Transduction experiments

Phage P1 grown on G11-R1a and G11-R1B1 was used in transduction of strain D11. Chloramphenicol resistance (100 µg./ml.) was used to select for transductants that had received R-factors. In both experiments 100 chloramphenicol-resistant transductants were purified and their resistance to ampicillin, chloramphenicol, streptomycin and sulfonamide determined by replica plating. There was no indication of any segregation of the resistance genes. Furthermore, transduction with P1 (G11-R1B1) gave the higher resistance characteristic for strains carrying the mutated R-factor while P1 (G11-R1a) gave the lower resistance level (see Table 4).

DISCUSSION

The results of the experiments described in this paper indicate that EMS induces mutations in the R-factor resulting in infective transfer of resistance to high drug concentrations. Similar findings have been reported for *Salmonella typhi* by Sompolinsky, Ben-Yakov, Aboud & Boldur (1967) but they do not discuss transfer of resistance to more than one antibiotic. Pearce & Meynell (1968) have described mutants of R1 giving increased resistance to streptomycin. In the work described in this paper resistance to four antibiotics were affected simultaneously. From Table 4 it can be concluded that the mutation in R1B1 increases the amount of resistance gene products per bacterium for all four resistance genes carried by R1a. Furthermore, genes involved in pair formation and gene transfer also seem to function more efficiently (Table 5).

The mutation could work either by altering the number of gene copies per cell or at the transcription level. The derepressed R-factor of Meynell & Datta (1967) did not cause any change in resistance pattern (Table 4). Fifty per cent of donor bacteria transfer this R-factor. Thus it is suggested that the R-factor genome can be functionally divided into at least two parts (operons?) of which the RTF (resistance transfer factor) has its own regulation. It is difficult to understand why the transfer of the R-factor should increase more than the transfer of chromosomal markers if the mutation through altered transcription increased piliation of Hfr bacteria.

An alteration in the number of gene copies per bacterium could be achieved in two ways, either by polymerization of the R-factor or by altered regulation of the replication of the R-factor resulting in an increased number of unchanged R1-factors per bacterium. EMS is known to induce chromosome aberrations of various kinds, e.g. mitotic recombination in yeast (Yost, Chaleff & Finerty, 1967), and it is possible that this mutagen may induce the formation of polymers of R-factors. Datta (1965) has shown that R1, which is of the *fi*⁺ type, codes for the same type of pili as does the F-factor. Thus once a mating pair was formed the probability of transferring the R-factor or the chromosome will most likely be a function of the number of copies of each replicon per bacterium. The transfer of the R-factor itself was increased more by the mutation giving R1B1 from R1a than the transfer of the chromosomal marker *proB*⁺ (Fig. 4, 5 and Table 5). Thus for R1B1 an increased number of free R-factor copies is more likely than a polymer R-factor. The results of the transduction experiments also favour this conclusion, as a polymer of R1a would presumably be too large to be transduced in one piece. Rownd, Nakaya & Nakamura (1966) have shown that the number of copies of R-factor per chromosome is different in different species.

Mutants such as R1B1 may be of value in studying the number of operons in R-factors and also of regulation of the number of episomes per bacterium.

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